many researchers and is leading to new ways to repair and regenerate cardiac disorders. Therapy by transplantation of bone marrow stem cells or tissue-derived stem cells into damaged cardiac tissue after myocardial infarction has been investigated (42) ; however, for children with severe congenital heart anomalies or lethal heart diseases like dilated cardiomyopathy, invasive therapies such as surgical operation or heart transplantation are still required. Further understanding of the regulatory factors during cardiac development is needed to enable less-invasive therapy for these patients.

In summary, our results suggest that MSTN, and its negative regulator, FLRG, could be involved in cardiac muscle development and valvulogenesis in cooperation with activin and FS.

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REFERENCES

1. Vale W, Rivier C, Hsueh A, Campen C, Meunier H, Bicsak T, Vaughan J, Corrigan A, Bardin W, Sawchenko P, Petraglia F, Plotsky P, Spiess J, Rivier J : Chemical and biological characterization of the inhibin family of protein hormones. Recent Prog Horm Res 44 : 1-34,1988
2. Chang H, Brown CW, Matzuk MM : Genetic analysis of the mammalian transforming growth factor-beta superfamily. Endocr Rev 23 : 787-823, 2002
3. Zimmerman CM, Padgett RW : Transforming growth factor beta signaling mediators and modulators. Gene 249 : 17-30, 2000
4. Sugino H, Tsuchida K : Activin and follistatin. In : Canalis E ed. Skeletal Growth Factors. Lippincott Williams & Wilkins, Philadelphia, 2000, pp.251-263
5. Tsuchida K, Arai KY, Kuramoto Y, Yamakawa N, Hasegawa Y, Sugino H : Identification and characterization of a novel follistatin-like protein as a binding protein for the TGF-β family. J Biol Chem 275 : 40788-40796, 2000
6. Tsuchida K, Matsuzaki T, Yamakawa N, Liu Z, Sugino H : Intracellular and extracellular control of activin function by novel regulatory molecules. Mol Cell Endorinol 180 : 25-31, 2001
7. Hayette S, Gadoux M, Martel S, Bertrand S, Tigaud I, Magaud JP, Rimokh R : FLRG (follistatin-related gene), a new target of chromosomal rearrangement in malignant blood disorders. Oncogene 16 : 2949-2954, 1998
8. Shimasaki S, Koga M, Esch F, Cooksey K, Mercado M, Koba A, Ueno N, Ying SY, Ling N, Guillemin R : Primary structure of the human follistatin precursor and its genomic organization. Proc Natl Acad Sci USA 85 : 4218-4222, 1988
9. Okabayashi K, Shoji H, Onuma Y, Nakamura T, Nose K, Sugino H, Asashima M : cDNA cloning and distribution of the Xenopus follistatin-related protein. Biochem Biophys Res Commun 254 : 42-48, 1999
10. de Winter JP, ten Dijke P, de Vries CJ, van Achterberg TA, Sugino H, de Waele P, Huylebroeck D, Verschueren K, van den Eijnden-van Raaij AJ : Follistatins neutralize activin bioactivity by inhibition of activin binding to its type II receptors. Mol Cell Endocrinol 116 : 105-114, 1996
11. Nakamura T, Sugino K, Titani K, Sugino H : Follistatin, an activin-binding protein, associates with heparan sulfate chains of proteoglycans on follicular granulose cells. J Biol Chem 266 : 19432-19437, 1991
12. Iemura S, Yamamoto TS, Takagi C, Uchiyama H, Natsume T, Shimasaki S, Sugino H, Ueno N : Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early Xenopus embyo. Proc Natl Acad Sci USA 95 : 9337-9342, 1998
13. Tortoriello DV, Sidis Y, Holzman DA, Holmes WE, Schneyer AL : Human follistatin-related protein : a structural homologue of follistatin with nuclear localization. Endocrinology 142 : 3426-3434, 2001
14. Lee SJ, and McPherron AC : Regulation of myostatin activity and muscle growth. Proc Natl Acad Sci USA 98 : 9306-9311, 2001
15. Zimmers TA, Davies MV, Koniaris LG, Haynes

P, Esquela AF, Tomkinson KN, McPherron AC, Wolfman NM, Lee SJ : Induction of cachexia in mice by systemically administered myostatin. Science 296 : 1486-1488, 2002
16. Hill JJ, Davies MV, Pearson AA, Wang JH, Hewick RM, Wolfman NM, Qiu Y : The myostatin propeptide and the follistatin-related gene are inhibitory binding proteins of myostatin in normal serum. J Biol Chem 277 : 40735-40741, 2002
17. McPherron AC, Lawler AM, Lee SJ : Regulation of skeletal muscle mass in mice by a new TGF-β superfamily member. Nature 387 : 83-90, 1997
18. Sharma M, Langley B, Bass J, Kambadur R : Myostatin in muscle growth and repair. Exerc Sport Sci Rev 29 : 155-158, 2001
19. Sharma M, Kambadur R, Matthews KG, Somers WG, Devlin GP, Conaglen JV, Fowke PJ, Bass JJ : Myostatin, a transforming growth factor-β superfamily member, is expressed in heart muscle and is upregulated in cardiomyocytes after infarct. J Cell Physiol 180 : 1-9, 1999
20. Cook SA, Matsui T, Li L, Rosenzweig A : Transcriptional effects of chronic Akt activation in the heart. J Biol Chem 277 : 22528-22533, 2002
21. Morissette MR, Cook SA, Foo S, McKoy G, Ashida N, Novikov M, Scherrer-Crosbie M, Li L, Mastui T, Brooks G, Rosenzweig A : Myostatin regulates cardiomyocyte growth through modulation of Akt signaling. Circ Res 99 : 15-24, 2006
22. Habara-Ohkubo A : Differentiation of beating cardiac muscle cells from a derivative of P19 embryonal carcinoma cells. Cell Struct Funct 21 : 101-110, 1996
23. Schaeren-Wiemers N, Gerfin-Moser A : A single protocol to detect transcripts of various types and expression levels in neural tissues and cultured cells : *in situ* hybridization using digoxigenin-labelled cRNA probes. Histochemistry 100 : 431-440, 1993
24. Hashimoto O, Kawasaki N, Tsuchida K, Shimasaki S, Hayakawa T, Sugino H : Difference between follistatin isoforms in the inhibition of activin signaling : activin neutralizing activity of follistatin isoforms is dependent on their affinity for activin. Cell Signal 12 : 565-571, 2000
25. Tsuchida K, Vaughan JM, Wiater E, Gaddy-Kurten D, Vale WW : Inactivation of activin-dependent transcription by kinase-deficient activin receptors. Endocrinology 136 : 5493-5503, 1995
26. Nakatani M, Yamakawa N, Matsuzaki T, Shimasaki S, Sugino H, Tsuchida K : Genomic organization and promoter analysis of mouse follistatin-related gene (FLRG). Mol Cell Endocrinol 189 : 117-123, 2002
27. Nakamura T, Sano M, Songyang Z, Schneider MD : A Wnt- and beta-catenin-dependent pathway for mammalian cardiac myogenesis. Proc Natl Acad Sci USA 100 : 5834-5839, 2003
28. Feijen A, Goumans MJ, van den Eijnden-van Raaij AJM : Expression of activin subunits, activin receptors and follistatin in postimplantation mouse embryo suggests specific developmental functions for different activins. Development 120 : 3621-3637, 1994
29. Tsuchida K, Nakatani M, Matsuzaki T, Yamakawa N, Liu Z, Bao Y, Arai KY, Murakami T, Takehara Y, Kurisaki A, Sugino H : Novel factors in regulation of activin signaling. Mol Cell Endocrinol 225 : 1-8, 2004
30. Bogdanovich S, Krag TO, Barton ER, Morris LD, Whittemore LA, Ahima RS, Khurana TS : Functional improvement of dystrophic muscle by myostatin blockade. Nature 420 : 418-421, 2002
31. Wang HQ, Takebayashi K, Tsuchida K, Nishimura M, Noda Y : Follistatin-related gene (FLRG) expression in human endometrium : sex steroid hormones regulate the expression of FLRG in cultured human endometrial stromal cells. J Clin Endocrinol Metab 88 : 4432-4439, 2003
32. Sugino K, Kurosawa N, Nakamura T, Takio K, Shimasaki S, Ling N, Titani K, Sugino H : Molecular heterogeneity of follistatin, an activin-binding protein. Higher affinity of the carboxyl-terminal truncated forms for heparan sulfate proteoglycans on the ovarian granulosa cell. J Biol Chem. 268 : 15579-15587, 1993
33. Hashimoto O, Nakamura T, Shoji H, Shimasaki S, Hayashi Y, Sugino H : A novel role of follistatin, an activin-binding protein, in the inhibition of activin action in rat pituitary cells. Endocytotic degradation of activin and its acceleration by follistatin associated with cell-surface heparan sulfate. J Biol Chem 272 : 13835-13842, 1997
34. Yatskievych TA, Ladd AN, Antin PB : Induction of cardiac myogenesis in avian pregastrula epiblast : the role of the hypoblast and activin. Development 124 : 2561-2570, 1997

35. Ladd AN, Yatskievych TA, Antin PB : Regulation of avian cardiac myogenesis by activin/TGF beta and bone morphogenetic proteins. Dev Biol 204 : 407-419, 1998
36. Shyu KG, Ko WH, Yang WS, Wang BW, Kuan P : Insulin-like growth factor-1 mediates stretch-induced upregulation of myostatin expression in neonatal rat cardiomyocytes. Cardiovasc Res 68 : 405-414, 2005
37. Amthor H, Nicholas G, McKinnell I, Kemp CF, Sharma M, Kambadur R, Patel K : Follistatin complexes Myostatin and antagonizes Myostatin-mediated inhibition of myogenesis. Dev Biol 270 : 19-30, 2004
38. Eisenberg LM, Markwald RR : Molecular regulation of atrioventricular valvuloseptal morphogenesis. Circ Res 77 : 1-6, 1995
39. Brown CB, Boyer AS, Runyan RB, Barnett JV : Requirement of type III TGF beta receptor for endocardial cell transformation in the heart. Science 283 : 2080-2082, 1999
40. Wang J, Sridurongrit S, Dudas M, Thomas P, Nagy A, Schneider MD, Epstein JA, Kaartinen V : Atrioventricular cushion transformation is mediated by ALK2 in the developing mouse heart. Dev Biol 286 : 299-310, 2005
41. Moore CS, Mjaatvedt CH, Gearhart JD : Expression and function of activin beta A during mouse cardiac cushion tissue formation. Dev Dyn 212 : 548-562, 1998
42. Fukuda K, Yuasa S : Stem cells as a source of regenerative cardiomyocytes. Circ Res 98 : 1002-1013, 2006

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A *Wnt3a* variant participates in chick apical ectodermal ridge formation: Distinct biological activities of Wnt3a splice variants in chick limb development

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Wnt/β-catenin signaling is involved in the formation of the apical ectodermal ridge (AER) during vertebrate limb development. Although Wnt3a is a potent ligand for chick AER formation, whether chick Wnt3a can induce *Fgf8* expression in chick embryos is unclear and the Wnt ligand involved in chick AER formation remains unknown. Here, we examined whether another Wnt3a isoform is expressed in the AER, and whether Wnt3 contributes to AER formation in chick as well as mouse embryos. We found that chick *Wnt3* was not expressed in the presumptive limb ectoderm at the early stages of AER formation. Using 5′-rapid amplification of cDNA ends, we isolated another chick *Wnt3a* transcript. This novel variant, Wnt3a variant 2, induced *Fgf8* in the limb ectoderm and activated the β-catenin pathway *in vivo* and *in vitro*. These data showed that Wnt3a variant 2 is an active form of chick Wnt3a that regulates chick AER formation.

Key words: alternative splicing, apical ectodermal ridge formation, chick, Wnt3, Wnt3a.

Introduction

Wnt/β-catenin signaling is involved in formation of the apical ectodermal ridge (AER) during vertebrate limb development. The Wnt ligand thought to trigger β-catenin signaling during chick limb development is Wnt3a, based on the following findings. *Wnt3a* expression is initially detected in the presumptive forelimb ectoderm at stage 15 and persists in the AER (Kengaku *et al.* 1997). *Wnt3a* is induced by Fgf10, a mesenchymal inducer for limb formation (Kawakami *et al.* 2001). Wnt3a overexpression induces the ectopic expression of *Fgf8* that is secreted from the AER and which acts as an AER factor (Kengaku *et al.* 1998). However, the latter studies used Wnt3a derived from mice. Whether or not chick Wnt3a can induce ectopic *Fgf8* expression during chick Wnt3a overexpression in the presumptive limb ectoderm is unclear. A key difference between chick and mouse Wnt3a is the absence and apparent presence, respectively, of a signal peptide used for secretion. A chimeric cWnt3a possessing the N-terminal signal peptide of mouse Wnt3a enhances the activation of β-catenin signaling (Galli *et al.* 2004). Thus, another Wnt isoform appears necessary to stimulate the AER inducer in chick embryos.

We found that *Wnt10a* is expressed in the surface ectoderm of presumptive limb fields before *Fgf8* expression is initiated and enhanced in the AER of the chick limb bud (Narita *et al.* 2005). The expression profiles of *Wnt10a* and Wnt3a are similar and Wnt10a overexpression induces ectopic *Fgf8* expression. However, *Wnt10a* is not induced by Fgf10 protein, suggesting that Wnt10a is probably not an AER inducer.

Wnt3 instead of *Wnt3a* is expressed in the developing limb buds of mouse embryos. Consistent with this expression, a conditional mutation of *Wnt3* disrupts AER formation and leads to limb defects (Barrow *et al.* 2003), whereas a loss of *Wnt3a* does not interfere with AER formation (Takada *et al.* 1994). Furthermore, the spontaneous mutation of *Wnt3* in humans causes tetra-amelia (Niemann *et al.* 2004). These data indicate that Wnt3 is an essential Wnt ligand for AER formation in both mouse and human embryos.

The present study examines Wnt3a and Wnt3 to define the Wnt ligand that acts during chick AER formation. We isolated chick *Wnt3* and tested whether cWnt3 contributes to AER formation in chick embryos. We then identified another transcript of chick *Wnt3a* and examined whether this transcript affects AER formation.

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Materials and methods

Isolation of chick Wnt3 cds and Wnt3a variant

We carried out 5′-rapid amplification of cDNA ends (RACE) according to the provided instruction manual (Invitrogen, Carlsbad, CA, USA) to isolate the 5′ sequence of the chick Wnt3 gene and another transcript of the chick Wnt3a gene. Total RNA was prepared from chicken embryos at stage 10. The sequences of chick Wnt3 and Wnt3a variant 2 (var. 2) were deposited to GenBank with the accession numbers EF068233 and EF068232, respectively.

RT-PCR analysis

Total RNA was isolated from different stages of whole chicken embryos, *Wnt3a* expression sites or at the following embryonic stages: primitive streak, whole embryos at stages 4–5; dorsal neural tube, neural tube at the forelimb level at stage 21; proximal otic vesicle, both side of otic vesicles at stage 21; AER and limb bud, fore- and hindlimb buds at stage 21; feather bud and dorsal dermis at stage 34 (E8). We synthesized cDNA using SuperScript II (Invitrogen) and random primers. The polymerase chain reaction (PCR) primers were:

Wnt3, upstream,
5′-AAGCCACACGGGAGTCTGCGTTT-3′;
Wnt3, downstream,
5′-CAGCTCACATAGCAGCACCAGTGGA-3′;
Wnt3a var. 1, upstream,
5′-TAGATCTGGTGGATGAAGTC-3′;
Wnt3a (BU205863), upstream,
5′-TTCTGGTGAGCGGGATGCAC-3′;
Wnt3a var. 2, upstream,
5′-GACAACTTTTCATCAATGGCCTC-3′;
Wnt3a variants downstream (common),
5′-GACCACCCAGCAGGTCTTCAC-3′;
Wnt3a common variants upstream,
5′-GGCTGTGACACTCGTCACAAGG-3′ and
downstream, 5′-TGCCCGCTTTATTTGCACG-3′.

Nucleic acids were denatured at 96°C for 2 min, then amplified by 10 cycles of 94°C for 30 s, 68°C for 30 s (Δ–0.5°C), 72°C for 2.5 min and 25 cycles of 94°C for 30 s, 63°C for 30 s, 72°C for 2.5 min followed by a final extension at 72°C for 5 min. The PCR products were separated in 2% agarose gels and stained with ethidium bromide.

Overexpression

Wild and retrovirus-free fertilized chicken eggs were infected (Line M, Nisseiken, Kobuchizawa, Japan) with retroviruses bearing the *Wnt* genes prepared as described by Narita *et al.* (2005). To overexpress the genes in limb ectoderm during AER formation, the retrovirus was injected into presumptive limb fields on the right side of stage 10–11 embryos. Two days later, the embryos were fixed in 4% paraformaldehyde (PFA) and examined by *in situ* hybridization. We injected the retrovirus into forelimb mesenchyme on the right side at stages 16–18 to achieve overexpression in limb mesenchymal cells. The embryos were fixed in formalin 7–8 days later and stained with Alcian green. To overexpress the genes in the dorsal ectoderm during feather formation, the retrovirus was injected into the dorsal dermis of the trunk region on the right side of stage 17 embryos. The embryos were fixed in 4% PFA 5 days later and *Shh* expression was analyzed using *in situ* hybridization.

Whole-mount in situ *hybridization*

A plasmid containing the entire coding region of chick *Wnt3* was digested with *Not*I and transcribed with SP6 RNA polymerase to prepare antisense probes. Antisense RNA probes for chicken *Fgf8* (Ohuchi *et al.* 1997), *Wnt3a (*common probe) (Kawakami *et al.* 2000) and *Shh* (Iseki *et al.* 1996; Wada *et al.* 1999) were synthesized as described. Embryos were fixed in 4% PFA in PBS at 4°C overnight and dehydrated in ethanol or methanol. Whole mount *in situ* hybridization proceeded as described (Kawakami *et al.* 1996). Embryos were stained with either BM purple or 4-nitroblue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP).

Detection of cytosolic β-catenin accumulation

Cytosolic β-catenin accumulation was assayed as described (Shimizu *et al.* 1997; Narita *et al.* 2005). Chicken embryonic fibroblasts (CEFs) were transfected with the replication-competent avian sarcoma (RCAS) virus constructs and then samples containing 50 μg of total protein were resolved by electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide (7.5%) gels. The primary antibody against β-catenin (BD Transduction Laboratory, San Diego, CA, USA) was diluted 1:2000. The protein was detected using the ECL Plus System (Amersham Pharmacia Biotech, Uppsala, Sweden) with a 1:10 dilution of the reagent mixture.

TOP/FOP reporter assay

Chicken embryonic fibroblasts were seeded into 24-well dishes at a density of 5.6×10^4 cells/well and cultured for 20 h. The cells were then cotransfected with 400 ng of RCAS construct (RCAS-Wnt3a var. 1, -Wnt3a var. 2, -mouse Wnt3a, -empty), 20 ng of reporter

plasmid (TOPFLASH, FOPFLASH) and 10 ng of internal control pRL-TK for 24 h. Luciferase activity was measured and normalized for transfection efficiency using the dual luciferase assay system (Promega, Madison, WI, USA). Each graph shows the average of four independent experiments.

Detection of HA-tagged Wnt3a proteins in medium

Wnt constructs containing a C-terminal hemagglutinin (HA) epitope tag were generated by PCR amplification and by cDNA fragment exchange. These genes were subcloned into the RCAS retrovirus vector and transfected into CEFs. Confluent cells were cultured in fresh Dulbecco's modified eagle's medium (DMEM) containing 5% fetal bovine serum (FBS) and 1% chicken serum for 4 days and then the medium was collected. Lysates were simultaneously prepared from the cells to confirm the translation of HA-tagged Wnt constructs. Conditioned medium (20 μL) and total cell lysate protein (50 μg) were resolved by electrophoresis on SDS-polyacrylamide (5–20%) gels and then examined by Western blotting using the primary anti-HA antibody (Roche, Mannheim, Germany) at a dilution of 1:2000. The protein was detected with a 1:10 dilution of the reagent mixture provided in the ECL Plus System (Amersham Pharmacia Biotech).

Cellular distribution of HA-tagged Wnt3a proteins

Chick embryonic fibroblasts were infected with RCAS HA-tagged Wnt viruses for 3 days, seeded onto glass cover slips for 12 h and fixed in PBS containing 4% PFA at 4°C for 30 min. The primary anti-HA antibody (Roche) and secondary antibody (fluorescein isothiocyanate [FITC] conjugate rat-IgG; Sigma) were diluted at 1:2000 and 1:1000, respectively. The endoplasmic reticulum (ER) was stained with Concanavalin A-Alexa Fluor 594 conjugate (Molecular Probes, Eugene, OR, USA) as an ER marker (Parkkinen *et al.* 1997). Cells were observed using a confocal laser-scanning microscope (Olympus, Tokyo, Japan).

Results

Expression of Wnt3 during chick AER formation

To examine whether Wnt3 contributes to chick AER formation, we cloned the entire coding sequence of chick *Wnt3* using 5′ RACE. A predicted chick Wnt3 proprotein consisting of 355 amino acids, possessed a signal peptide for secretion and was 96% and 84% identical to mouse Wnt3 and Wnt3a, respectively (Fig. 1A; accession number: EF068233). We showed using reverse transcription (RT)-PCR that *Wnt3* is expressed in the neural tube, and fore- and hindlimb buds at embryonic stage 21, and in the dorsal skin at stage 34 (Fig. 1B). Whole-mount *in situ* hybridization revealed *Wnt3* expression in the brain, neural tube and ectoderm of the branchial arch (Fig. 1C–F), but not in the ectoderm of the fore- and hindlimbs of the limb bud during the early stages of AER formation (Fig. 1C). However, Wnt3 was initially expressed in the ectoderm of the hindlimb at stage 21 (Fig. 1E,F). These results suggested that Wnt3 does not contribute to chick AER formation.

Isolation of chick Wnt3a variants

Two chick *Wnt3a* transcripts have been recorded in GenBank under accession numbers AB024080 and BU20586 (Kawakami *et al.* 2000 and Boardman *et al.* 2002, respectively). Both isoforms have essentially identical C-terminal regions and differ in the N-terminal sequence (Fig. 1G, Red arrows). Unlike mouse Wnt3a, neither of the isoforms has N-terminal hydrophobic signal peptides.

To obtain a third isoform possessing the signal peptide, we carried out 5′ RACE and isolated a fragment with considerable identity to the amino-terminal region of mouse Wnt3a. The predicted protein of the new isoform comprised 352 amino acid residues including an 18-residue signal peptide at the N-terminus, and was 88.1% identical to mouse Wnt3a protein (Fig. 1G). We compared the expression profiles of the three *Wnt3a* variants by RT-PCR using specific primers (Fig. 1H). The new isoform was expressed in the primitive streak, dorsal neural tube, proximal otic vesicle, the AER and the epithelium of feather buds. *Wnt3a* (AB024080) was similarly expressed, except for the feather buds, whereas *Wnt3a* (BU205863) was faintly expressed in all tissues. When we searched the chick genome database, we found that the 5′ sequence of Wnt3a (BU205863) corresponded to the intron sequence of Wnt3a (AB024080) and the new isoform (data not shown). Thus, Wnt3a (BU205863) appears to be a pre-mRNA product of other Wnt3a isoforms. We confirmed the spatial expression of Wnt3a. *Wnt3a* was expressed in the primitive streak edge, dorsal neural tube, proximal otic vesicle, the AER and epithelium of feather buds, as described (Hollyday *et al.* 1995; Stevens *et al.* 2003; Chang *et al.* 2004; Fig. 1I–K).

Novel Wnt3a isoform is active in chick embryos

We carried out functional assays of Wnt3a (AB024080) and the new isoform. To unambiguously discriminate these chick *Wnt3a* transcripts, we designated Wnt3a

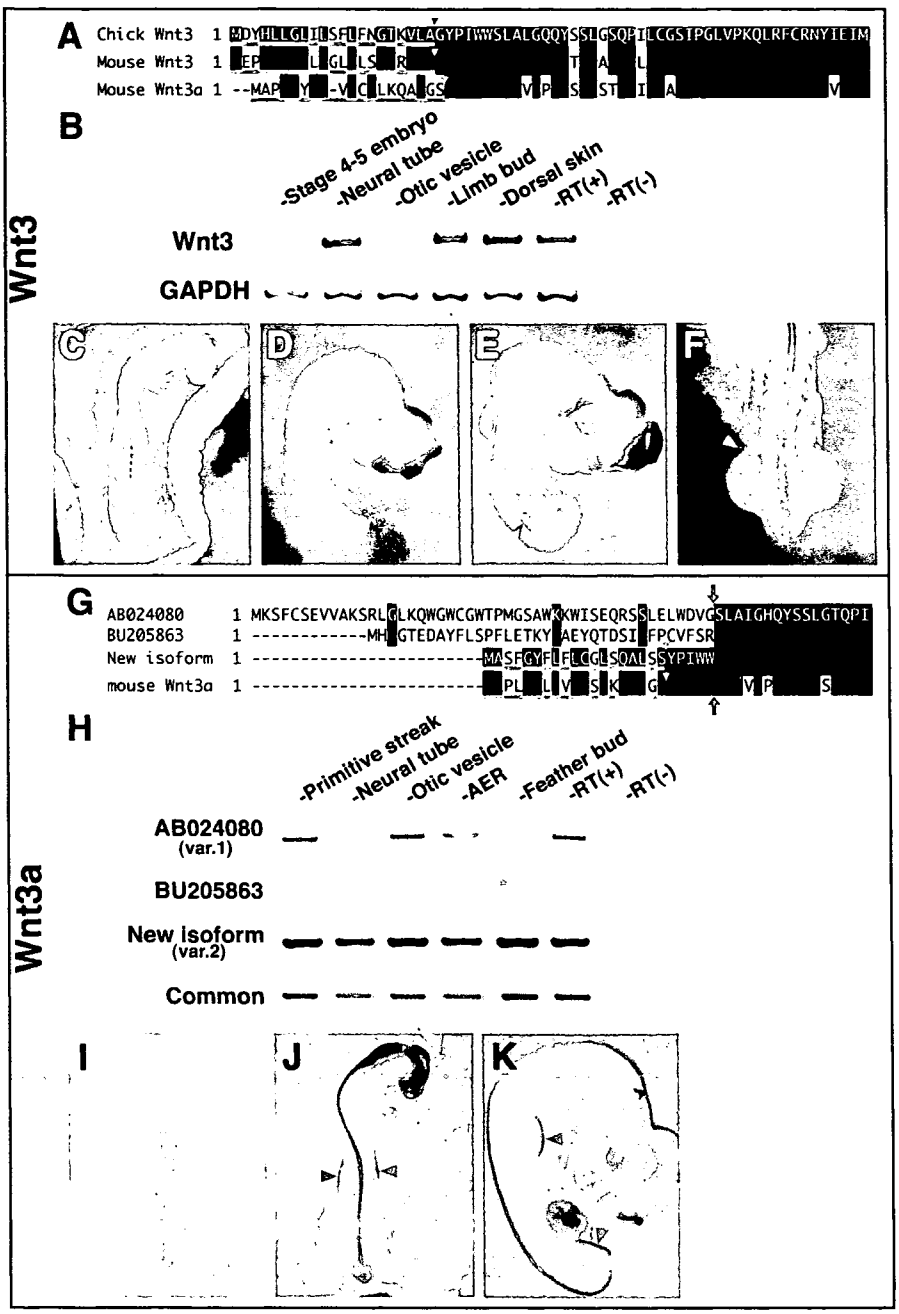

Fig. 1. Wnt3 and Wnt3a in chick embryos. (A) Chick Wnt3 amino acid sequence. Blue arrowhead shows prospective cleavage site of signal peptide (Green underline). (B) *Wnt3* expression by reverse transcription-polymerase chain reaction (RT-PCR). (C–E) Wnt3 expression during apical ectodermal ridge (AER) formation of chick embryos at stages 15 (C), 19 (D) and 21 (E, F). *Wnt3* was expressed in the anterior proximal ectoderm of stage 21 embryos, but not in developing AERs (E, Arrowhead). (F) High magnification view of E. (G) Comparison of chick Wnt3a variants and mouse Wnt3a at N-terminal region. Novel Wnt3a isoform is closely related to mouse Wnt3a. Conserved amino acids are shown as closed boxes. Green underline and blue arrowhead show signal peptide and its predicted cleavage site, respectively. (H) Expression of chick Wnt3a variants determined by RT-PCR. (I-K) Whole-mount *in situ* hybridization using a probe containing chicken Wnt3a common sequence. *Wnt3a* expression at stages 5 (I), 17 (J) and 22 (K). *Wnt3a* is expressed in primitive streak edge (I), dorsal side of neural tube, proximal part of otic vesicle and AER (K; red arrowheads).

(AB024080) as Wnt3a variant 1 (Wnt3a var. 1) and the new Wnt3a isoform (EF068232) as Wnt3a variant 2 (Wnt3a var. 2) (Table 1).

We overexpressed Wnt3a variants in the limb ectoderm to determine whether Wnt3a var. 2 contributes to AER formation. Wnt3a var. 2 overexpression induced the ectopic expression of *Fgf8* lateral to that of endogenous *Fgf8* (Fig. 2B; 5/11 embryos). Wnt3a var. 1 and control overexpression did not lead to ectopic *Fgf8* expression (Fig. 2A,C). These results showed that Wnt3a var. 2, but not var. 1, is involved in chick AER formation.

The forced activation of Wnt/β-catenin signaling affects gene expression and morphogenesis in limbs and feather buds (Hartmann & Tabin 2000; Chang *et al.* 2004). To test whether Wnt3a var. 2 is involved in these tissues, we overexpressed Wnt3a variants in the limb mesoderm and then in the dorsal ectoderm during feather bud formation. The overexpression of Wnt3a var. 2 in the limb mesoderm led to limb truncation compared with the control (Fig. 2E,F), whereas that of Wnt3a var. 1 did not affect limb extension (Fig. 2D). The overexpression Wnt3a var. 2 in the dorsal ectoderm resulted in fusion or expansion

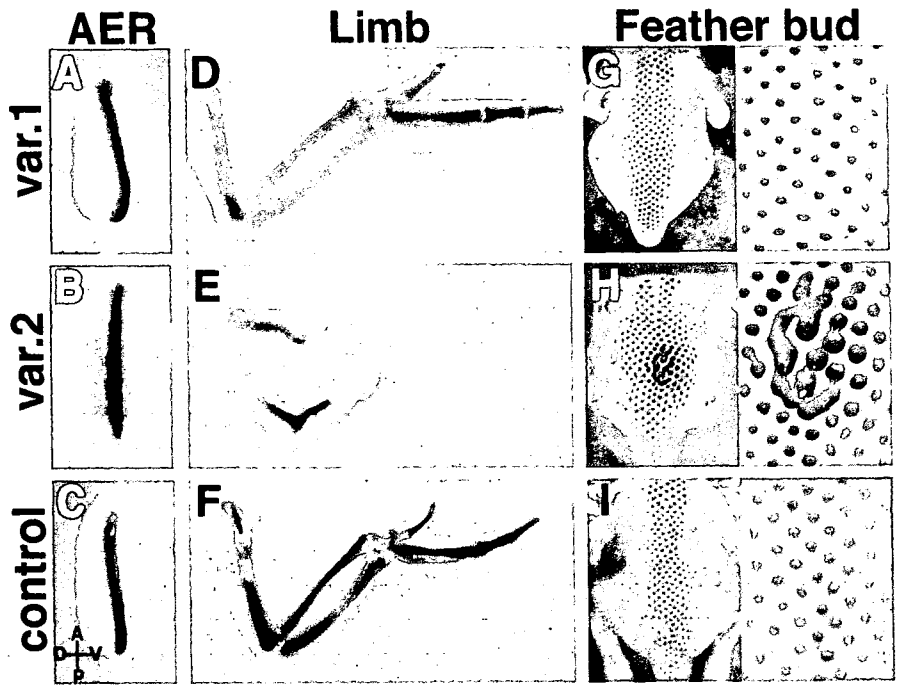

Fig. 2. Wnt3a variant 2 is an active form. (A–C) Presumptive limb ectoderm was infected with virus at the embryonic stage 10–12 and *Fgf8* expression was analyzed at stages 23–24. Top, anterior; left, dorsal. (A) Wnt3a var. 1 overexpression does not affect *Fgf8* expression. (B) Wnt3a var. 2 overexpression leads to ectopic *Fgf8* expression lateral to apical ectodermal ridge (AER) endogenously expressing *Fgf8* (5/11). Light blue indicates cells infected replication-competent avian sarcoma (RCAS) virus. (C) *Fgf8* expression in forelimb bud of control embryo at stage 23. (D–F) Limb mesenchyme injected with virus at embryonic stage 16 and analyzed around stage 36 (E10). Forelimb is stained with Alcian green to visualize bone structures. Top, anterior; left, proximal. (D) Wnt3a var. 1 overexpression results in normal limb development. (E) Wnt3a var. 2 overexpression results in abnormal bone structure and truncated limbs (4/4). (F) Control embryo at stage 36 (E10). (G–I) *Shh* expression in the dorsal epithelium. Top, anterior. Dorsal view. High magnification views of G–I are on the right side. (G–I) Dorsal skin was injected with virus at the embryonic stage 17 and analyzed at stages 34–35 (E8–9). (G) Wnt3a var. 1 overexpression results in normal *Shh* expression. (H) Wnt3a var. 2 overexpression leads to extension and fusion of *Shh* expression domain. (I) Uninjected wild embryo at stage 34 (E8).

Table 1. Alternative transcripts of chick Wnt3a

Name	Accession no.	Signal peptide
Wnt3a variant 1	AB024080	–
–	BU205863	–
Wnt3a variant 2	EF068232	+

Wnt3a variant 2 has been independently isolated with a *cWnt3a* variant by Fokina and Frolova (2006; GenBank accession number DQ022307).

of *Shh* expression in the feather buds (Fig. 2H) whereas that of Wnt3a var. 1 and the control did not affect *Shh* expression (Fig. 2G,I). These data demonstrated that Wnt3a var. 2 is active in developing chick embryos.

Wnt3a var. 2 can activate β-catenin signaling

We tested whether Wnt3a var. 2 can activate β-catenin signaling *in vitro* using the TOP/FOP reporter assay (Korinek *et al.* 1997). Wnt and reporter constructs were cotransfected into chicken embryonic fibroblasts (CEF) and luciferase activity was measured 24 h later. Wnt3a var. 2 and mouse Wnt3a, a positive control, significantly increased reporter activity, whereas Wnt3a var. 1 did not (Fig. 3A). We examined by Western blotting whether Wnt3a var. 2 accumulates cytosolic β-catenin. The Wnt3a var. 1, var. 2 or mouse Wnt3a construct was overexpressed in CEFs, and then we analyzed β-catenin protein in the cell membrane and cytosol fractions. Again, the overexpression of Wnt3a var. 2, as well as mouse Wnt3a, resulted in cytosolic β-catenin accumulation, whereas that of Wnt3a var. 1 did not (Fig. 3B).

We questioned whether Wnt3a var. 2 protein is secreted into the extracellular space and if so, whether it interacts with receptors on target cells. We introduced a hemagglutinin (HA) epitope tag at the C-terminus to detect Wnt3a proteins. HA-tagged Wnt3a var. 1 and var. 2 were transfected into CEFs and culture media were collected 4 days later. Tagged Wnt3a var. 2 protein was secreted into culture medium whereas HA-tagged Wnt3a var. 1 protein was undetectable (Fig. 3C). We also examined the intracellular distribution of Wnt proteins in cells that expressed a construct tagged with HA. Some HA-tagged Wnt3a var. 2 positive vesicles were localized inside the ER (Fig. 3D, lower right, white arrowheads), suggesting that Wnt3a var. 2 protein is present in the secretion pathway. In contrast, HA-tagged Wnt3a var. 1 protein was uniformly distributed within the cytosol (Fig. 3D, upper panels).

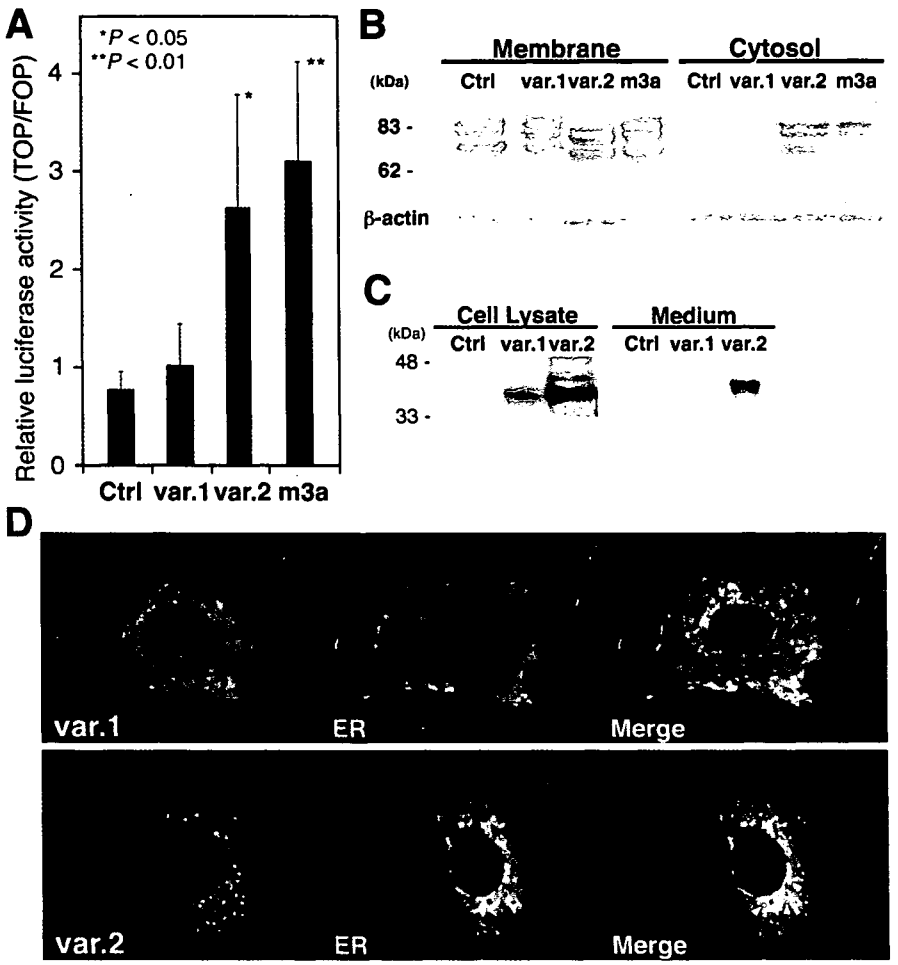

Fig. 3. Wnt3a variant 2 (var. 2) activates β-catenin signaling pathway. (A) TOP/FOP-FLASH reporter assay. Wnt3a var. 2 increased luciferase activity like mouse Wnt3a (m3a), whereas Wnt3a var. 1 (var. 1) did not affect luciferase activity. Each bar graph shows an average of four independent experiments. Ctrl, control chicken embryonic fibroblasts (CEFs) transfected with replication-competent avian sarcoma (RCAS) virus empty construct; var. 1, Wnt3a variant 1; var. 2, Wnt3a variant 2; m3a, mouse Wnt3a. (B) Western blots of β-catenin in cytosolic and membrane fractions of CEFs expressing Wnt proteins. β-actin indicates loading control. Left, molecular weight marker protein. Ctrl, control CEFs alone. (C) Western blots of Wnt proteins in conditioned media from CEFs expressing hemagglutinin (HA)-tagged Wnt proteins. HA-tagged Wnt3a var. 2 is secreted in conditioned medium, whereas HA-tagged Wnt3a var. 1 protein is virtually undetectable. Left, molecular weight marker protein. Ctrl, control CEFs alone. (D) Distribution of HA-tagged Wnt3a var. 1 and var. 2 proteins in CEF. Wnt3a var. 1 (upper panels). Wnt3a var. 2 (lower panels). HA-tagged Wnt3a (left panels, green). Endoplasmic reticulum (ER) stained with Concanavalin A as ER marker (middle panels, magenta). Wnt3a-HA is merged with ER staining (right panels). Some Wnt3a var. 2-HA positive vesicles are localized in ER (lower right, white arrowheads), suggesting that Wnt3a var. 2-HA is present in the secretion pathway. Wnt3a var. 2 is also visible as bright spots outside the ER (lower right, green). These vesicles may be aggresomes in which misfolded and aggregated proteins under forced expression are stored (Garcia-Mata, R *et al.* 2002).

Collectively, these results show that Wnt3a var. 2 is secreted and induces β-catenin accumulation in cells where it regulates the transcription of target genes mediated by Lef/Tcf transcriptional factors. In contrast, Wnt3a var. 1 that lacks a signal peptide for secretion is retained within cells and does not contribute to developmental processes through the activation of β-catenin signaling.

Discussion

We isolated another chick *Wnt3a* transcript named Wnt3a var. 2 that was generated by an alternative first exon and which possessed a signal peptide for secretion (Fig. 1G). During chick development, *Wnt3a var. 2* is expressed in the primitive streak, dorsal neural tube, proximal otic vesicle, the AER and the feather bud in which *Wnt3a* is expressed (Hollyday *et al.* 1995; Stevens *et al.* 2003; Chang *et al.* 2004; Fig. 1H–K). We found that Wnt3a var. 2 overexpression affected morphogenetic events in chick embryos (Fig. 2B,E,H) and that Wnt3a var. 2 activated β-catenin signaling *in vitro* (Fig. 3A,B). Thus, Wnt3a var. 2 is an active isoform of chick Wnt3a.

Alternatively spliced variants of Wnt3a

The chick genome project has revealed that the chick *Wnt3a* gene comprises five exons that span over 87 kb on the second chromosome. Transcriptional variants of the *Wnt3a* gene are generated by an alternative first exon (Fig. 4A). Exon 1a that generates Wnt3a var. 1 is located about 1 kb upstream of exon 1b that generates Wnt3a var. 2. Whereas Wnt3a var.

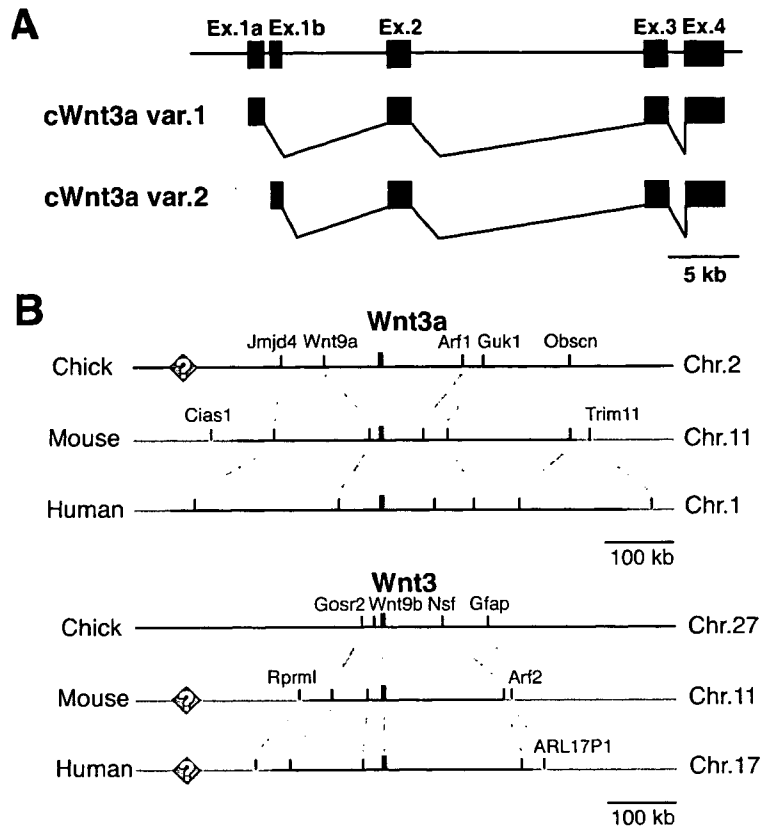

Fig. 4. Genomic organization of Wnt3 and Wnt3a. (A) Structure of chick *Wnt3a* gene. Chick *Wnt3a* gene has five exons (closed boxes), the sizes of which are emphasized. (B) Location of *Wnt3a* and *Wnt3* genes in chick, mouse and human genome. Upstream and downstream regions of *Wnt3a* and *Wnt3* locus are conserved among chick, mouse and human genomes. *Wnt3a* and *Wnt9a* genes are located in a head-to-head manner in chick chromosome 2, mouse chromosome 11B1.3 and human chromosome 1q42. *Wnt3* and *Wnt9b* genes are similarly clustered in chick chromosome 27, mouse chromosome 11E1 and human chromosome 17q21. Human ARL17P is a pseudogene of mouse *Arf2* gene (gray dotted line). Red rhombus indicates a putative limb-specific regulatory element that might be located distantly in both genes, like that of *Sonic hedgehog*, which is located about 800 kb from the coding sequence (Ianakiev *et al.* 2001; Sagai *et al.* 2004, 2005).

1 is unique to the chick, Wnt3a var. 2 is conserved among vertebrates. The alternative splicing of the first exon is the same for human *Wnt16* (Fear *et al.* 2000). The unique isoform in humans also lacks a signal peptide.

We compared chick, *Xenopus tropicalis* and Zebrafish genomic sequences to determine how the additional exon 1a of chick Wnt3a is generated. Genomic sequences within 1.5 kb upstream of exon 1b were conserved, whereas sequences further than 1.5 kb upstream from exon 1b, including the region around exon 1a, were not conserved among these animals. A foreign DNA fragment including exon 1a might be inserted into the genomic sequence upstream of exon 1b. The *Wnt* genes (*int-1* and *int-4*) were originally identified as a proto-oncogene that the mouse mammary tumor virus (MMTV) integrates into the promoter region (Nusse 1988; Roelink *et al.* 1990). A movable DNA fragment might be inserted into the promoter region of the *Wnt* gene family at a high probability.

The roles of Wnt3a var. 1 during chick development remain unclear. Wnt3a var. 1 did not interfere with β-catenin signaling by Wnt3a var. 2 and Wnt3a var. 1 overexpression did not affect limb and feather bud formation (Fig. 2A,D,G). In addition, when both *Wnt3a var. 1*and *var. 2* mRNAs were coinjected into one-cell-stage amphibian embryos, Wnt3a var. 1 did not affect *xNr3* expression through β-catenin signaling by Wnt3a var. 2 (data not shown). Although Wnt3a var. 1 might play many biological roles, *Wnt3a var. 1* might affect *Wnt3a var. 2* transcription. The expression profiles of *Wnt3a var. 1* and *Wnt3a var. 2* were almost identical (Fig. 1H), suggesting that these variants share promoters for transcription into mRNAs. When *Wnt3a var. 1* mRNA is transcribed, the relative levels of the *var. 2* mRNA might be decreased and var. 2 production should be down-regulated. Further analyses using gene manipulation such as the Cre-LoxP system are required to reveal the function of *Wnt3a var. 1*.

Wnt3a and Wnt3 in AER formation

Wnt/β-catenin signaling is necessary for AER formation during vertebrate limb development. Wnt3 is the Wnt ligand involved in mouse AER formation. *Wnt3* is widely expressed in the limb ectoderm and the conditional knockout of Wnt3 leads to disrupted *Fgf8* expression in the AER (Barrow *et al.* 2003). We found that *Wnt3* was not expressed in limb ectoderm at the early stages of AER formation, suggesting that Wnt3 does not contribute to this process in the chick (Fig. 1A–F). In contrast, *Wnt3a* is expressed in the prospective limb ectoderm before *Fgf8* expression and persists in the AER (Kengaku *et al.* 1997; Fig. 1H,J,K).

We found that Wnt3a (var. 2) overexpression induced ectopic *Fgf8* expression in the developing chick limb (Fig. 2B). Thus, Wnt3a is involved in chick AER formation.

Wnt3 and Wnt3a in chick and mouse embryos

When compared with the expression of Wnt3a and Wnt3 in chick and mouse embryos, that of *Wnt3a* and *Wnt3* in the limb ectoderm differed between these embryos. *Wnt3a* was expressed in the primitive streak, central nervous system and otic vesicle of both chick and mouse embryos, but in the limb ectoderm and feather bud of only chick embryos (Parr *et al.* 1993; Takada *et al.* 1994; Hollyday *et al.* 1995; Kengaku *et al.* 1997, 1998; Stevens *et al.* 2003; Chang *et al.* 2004). *Wnt3* was similarly expressed in the central nervous system, branchial arch and dorsal ectoderm of both chick and mouse embryos, but in the limb ectoderm during AER formation in only mouse embryos (Roelink & Nusse 1991; Salinas & Nusse 1992; Bulfone *et al.* 1993; Parr *et al.* 1993; Liu *et al.* 1999; Barrow *et al.* 2003; Robertson *et al.* 2004). We considered how the differences between chick and mouse Wnt ligands with respect to AER formation arise. *Wnt3a* and *Wnt3* are expressed in chick and mouse limb ectoderm, respectively, indicating that a limb-specific *cis*-regulatory element is located upstream of the chick *Wnt3a* and mouse *Wnt3* genes (Fig. 4B, Rhombus). *Wnt3a* and *Wnt3* gene organization is conserved among vertebrates, demonstrating that *Wnt3a* and *Wnt3* are close paralogs that arise from duplication of a progenitor *Wnt3a/3* gene (Sidow 1992; Fig. 4B). The progenitor *Wnt3a/3* gene might have had a limb-specific element before duplication into *Wnt3a* and *Wnt3* that was lost during the evolution of chicks and mice, respectively. Alternatively, chick *Wnt3a* and mouse *Wnt3* might have independently acquired a limb-specific *cis*-regulatory element. Snail and Slug expression profiles are interchangeable between the chick and the mouse. Snail in the mouse and Slug in the chick is expressed in precursors of the mesoderm and the neural crest, and also in migratory populations (Sefton *et al.* 1998; Nieto 2002). Nieto (2002) proposed that the expression of Snail and Slug interchanges under conditions of the differential loss of a tissue-specific *cis*-regulatory element and the differential availability of an upstream regulator. Chick Wnt3a and mouse Wnt3 in AER formation also provide important clues to the mechanism of interchanges in expression profiles.

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References

Barrow, J. R., Thomas, K. R., Boussadia-Zahui, O. *et al.* 2003. Ectodermal *Wnt3*/β-catenin signaling is required for the establishment and maintenance of the apical ectodermal ridge. *Genes Dev.* **17**, 394–409.

Boardman, P. E., Sanz-Ezquerro, J., Overton, I. M. *et al.* 2002. A Comprehensive Collection of Chicken cDNAs. *Curr. Biol.* **12**, 1965–1969.

Bulfone, A., Puelles, L., Porteus, M. H. *et al.* 1993. Spatially restricted expression of Dlx-1, Dlx-2 (Tes-1), Gbx-2, and Wnt-3 in the embryonic day 12.5 mouse forebrain defines potential transverse and longitudinal segmental boundaries. *J. Neurosci.* **13**, 3155–3172.

Chang, C. H., Jiang, T. X., Lin, C. M., Burrus, L. W., Chuong, C. M. & Widelitz, R. 2004. Distinct Wnt members regulate the hierarchical morphogenesis of skin regions (spinal tract) and individual feathers. *Mech. Dev.* **121**, 157–171.

Fear, M. W., Kelsell, D. P., Spurr, N. K. & Barnes, M. R. 2000. Wnt-16a, a novel Wnt-16 isoform, which shows differential expression in adult human tissues. *Biochem. Biophys. Res. Commun.* **278**, 814–820.

Fokina, V. M. & Frolova, E. I. 2006. Expression patterns of Wnt genes during development of an anterior part of the chicken eye. *Dev. Dyn.* **235**, 496–505.

Galli, L. M., Willert, K., Nusse, R. *et al.* 2004. A proliferative role for Wnt-3a in chick somites. *Dev. Biol.* **269**, 489–504.

Garcia-Mata, R., Gao, Y. S. & Sztul, E. 2002. Hassles with taking out the garbage: aggravating aggresomes. *Traffic.* **3**, 388–396.

Hartmann, C. & Tabin, C. J. 2000. Dual roles of Wnt signaling during chondrogenesis in the chicken limb. *Development* **127**, 3141–3159.

Hollyday, M., McMahon, J. A. & McMahon, A. P. 1995. Wnt expression patterns in chick embryo nervous system. *Mech. Dev.* **52**, 9–25.

Ianakiev, P., van Baren, M. J., Daly, M. J. *et al.* 2001. Acheiropodia is caused by a genomic deletion in C7orf2, the human orthologue of the *Lmbr1* gene. *Am. J. Hum. Genet.* **68**, 38–45.

Iseki, S., Araga, A., Ohuchi, H. *et al.* 1996. Sonic hedgehog is expressed in epithelial cells during development of whisker, hair, and tooth. *Biochem. Biophys. Res. Commun.* **218**, 688–693.

Kawakami, Y., Ishikawa, T., Shimabara, M. *et al.* 1996. BMP signaling during bone pattern determination in the developing limb. *Development* **122**, 3557–3566.

Kawakami, Y., Wada, N., Nishimatsu, S. & Nohno, T. 2000. Involvement of frizzled-10 in Wnt-7a signaling during chick limb development. *Dev. Growth Differ.* **42**, 561–569.

Kawakami, Y., Capdevila, J., Buscher, D., Itoh, T., Rodriguez-Esteban, C. & Izpisua-Belmonte, J. C. 2001. WNT signals control FGF-dependent limb initiation and AER induction in the chick embryo. *Cell* **104**, 891–900.

Kengaku, M., Twombly, V. & Tabin, C. 1997. Expression of Wnt and Frizzled genes during chick limb bud development. *Cold Spring Harb. Symp Quant. Biol.* **62**, 421–429.

Kengaku, M., Capdevila, J., Rodriguez-Esteban, C. *et al.* 1998. Distinct WNT pathways regulating AER formation and dorsoventral polarity in the chick limb bud. *Science* **280**, 1274–1277.

Korinek, V., Barker, N., Morin, P. J. *et al.* 1997. Constitutive transcriptional activation by a β-catenin-Tcf complex in APC-/- colon carcinoma. *Science* **275**, 1784–1787.

Liu, P., Wakamiya, M., Shea, M. J., Albrecht, U., Behringer, R. R. & Bradley, A. 1999. Requirement for Wnt3 in vertebrate axis formation. *Nat. Genet.* **22**, 361–365.

Narita, T., Sasaoka, S., Udagawa, K. *et al.* 2005. Wnt10a is involved in AER formation during chick limb development. *Dev. Dyn.* **233**, 282–287.

Niemann, S., Zhao, C., Pascu, F. *et al.* 2004. Homozygous WNT3 mutation causes tetra-amelia in a large consanguineous family. *Am. J. Hum. Genet.* **74**, 558–563.

Nieto, M. A. 2002. The snail superfamily of zinc-finger transcription factors. *Nat. Rev. Mol. Cell Biol.* **3**, 155–166.

Nusse, R. 1988. The *int* genes in mammary tumorigenesis and in normal development. *Trends Genet.* **4**, 291–295.

Ohuchi, H., Shibusawa, M., Nakagawa, T. *et al.* 1997. A chick *wingless* mutation causes abnormality in maintenance of *Fgf8* expression in the wing apical ridge, resulting in loss of the dorsoventral boundary. *Mech. Dev.* **62**, 3–13.

Parkkinen, J. J., Lammi, M. J., Agren, U. *et al.* 1997. Polyamine-dependent alterations in the structure of microfilaments, Golgi apparatus, endoplasmic reticulum, and proteoglycan synthesis in BHK cells. *J. Cell Biochem.* **66**, 165–174.

Parr, B. A., Shea, M. J., Vassileva, G. & McMahon, A. P. 1993. Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. *Development* **119**, 247–261.

Robertson, C. P., Braun, M. M. & Roelink, H. 2004. Sonic hedgehog patterning in chick neural plate is antagonized by a Wnt3-like signal. *Dev. Dyn.* **229**, 510–519.

Roelink, H. & Nusse, R. 1991. Expression of two members of the Wnt family during mouse development – restricted temporal and spatial patterns in the developing neural tube. *Genes Dev.* **5**, 381–388.

Roelink, H., Wagenaar, E., Lopes da Silva, S. & Nusse, R. 1990. Wnt-3, a gene activated by proviral insertion in mouse mammary tumors, is homologous to *int-1/Wnt-1* and is normally expressed in mouse embryos and adult brain. *Proc. Natl. Acad. Sci. USA* **87**, 4519–4523.

Sagai, T., Masuya, H., Tamura, M. *et al.* 2004. Phylogenetic conservation of a limb-specific, cis-acting regulator of Sonic hedgehog (*Shh*). *Mamm. Genome.* **15**, 23–34.

Sagai, T., Hosoya, M., Mizushina, Y., Tamura, M. & Shiroishi, T. 2005. Elimination of a long-range *cis*-regulatory module causes complete loss of limb-specific *Shh* expression and truncation of the mouse limb. *Development* **132**, 797–803.

Salinas, P. C. & Nusse, R. 1992. Regional expression of the Wnt-3 gene in the developing mouse forebrain in relationship to diencephalic neuromeres. *Mech. Dev.* **39**, 151–160.

Sefton, M., Sanchex, S. & Nieto, M. A. 1998. Conserved and divergent roles for members of the *Snail* family of transcription factors in the chick and mouse embryo. *Development* **125**, 3111–3121.

Shimizu, H., Julius, M. A., Giarre, M., Zheng, Z., Brown, A. M. & Kitajewski, J. 1997. Transformation by Wnt family proteins correlates with regulation of beta-catenin. *Cell Growth Differ.* **8**, 1349–1358.

Sidow, A. 1992. Diversification of the *Wnt* gene family on the ancestral linage of vertebrates. *Proc. Natl. Acad. Sci. USA* **89**, 5098–5102.

Stevens, C. B., Davies, A. L., Battista, S., Lewis, J. H. & Fekete, D. M. 2003. Forced activation of Wnt signaling alters morphogenesis and sensory organ identity in the chicken inner ear. *Dev. Biol.* **261**, 149–164.

Takada, S., Stark, K. L., Shea, M. J., Vassileva, G., McMahon, J. A. & McMahon, A. P. 1994. Wnt-3a regulates somite and tailbud formation in the mouse embryo. *Genes. Dev.* **8**, 174–189.

Wada, N., Kawakami, Y. & Nohno, T. 1999. Sonic hedgehog signaling during digit pattern duplication after application of recombinant protein and expressing cells. *Dev. Growth Differ.* **41**, 567–574.

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Nuclear localization of beta-catenin involved in precancerous change in oral leukoplakia

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Abstract

Background: Oral leukoplakia is a precancerous change developed in the oral mucosa, and the mechanism that oral leukoplakia becomes malignant through atypical epithelium is not known. Here we compared the β-catenin expression detected by immunohistochemical staining in the normal oral epithelium and in the oral leukoplakia with or without dysplasia.

Results: The normal oral epithelium showed β-catenin expression only in the cell membrane, but not in the nuclei. In the oral leukoplakia without dysplasia, 7 out of 17 samples (41%) showed β-catenin expression in the cell membrane, and 5 samples (29%) showed expression in the nuclei. In the oral leukoplakia with dysplasia, nuclear expression of β-catenin was shown in 11 out of 12 samples (92%). Incidence of nuclear β-catenin expression was significantly different between dysplasia and normal oral epithelium ($P < 0.01$), and also between oral leukoplakia with dysplasia and those without dysplasia ($P < 0.01$). Wnt3 expression was detected in the epithelial cell membrane or cytoplasm in oral leukoplakia where nuclear expression of β-catenin was evident, but not in epithelial cells without nuclear expression of β-catenin.

Conclusion: The components of canonical Wnt pathway, such as Wnt3, β-catenin, and cyclin D1, were detected, implying that this pathway is potentially involved in the progression of dysplasia in oral leukoplakia.

Background

Oral leukoplakia is known as a precancerous change of squamous cell carcinoma developed in oral mucosa [1-3]. Leukoplakia is a term expressing clinical disease state, and it occurs in every intra-oral locus and shows various observations. Because a clinician is difficult to be settled with precancerous lesion in these, we require histopathology examination. Leukoplakia diagnosed as epithelial dysplasia histopathology in leukoplakia becomes precancerous. It is said that it may become malignant transformation in progression of the severity in epithelial dysplasia [4]. Although much works have been done on the leukoplakia, the mechanism that oral mucosa epithelium constituting leukoplakia becomes malignant through atypical epithelium is not known.

There are many reports on β-catenin accumulation into a nucleus of a cancer cell in the epithelial malignant tumor, including colorectal cancer [5-8], and transcription activation of a target gene by β-catenin/TCF is known to participate in malignant transformation [9,10]. Transcription activation by β-catenin is triggered by binding of Wnt family to the cell membrane receptors, called Frizzled and LRP5/6, which transduces Wnt signal inside the cell through inactivation of GSK-3. APC and Axin constitute a complex to phosphorylate β-catenin with GSK-3. In the presence of Wnt, the receptor complex transduces negative signals to APC/Axin/GSK-3 complex through Dishevelled, and thus GSK-3 becomes unable to phosphorylate cellular β-catenin. Accumulated β-catenin in cytoplasm is now translocated into a nucleus, forms a transcriptional activation complex with TCF/LEF1 [10-13], and activates various target genes such as c-myc and cyclin D1 [14-17]. In the absence of Wnt, β-catenin in cytoplasm is immediately phosphorylated by GSK-3/Axin/APC complex and receives ubiquitination, eventually leading to degradation in proteasome, and thus disappeared in cytoplasm immediately [9,10].

Because Wnt family is known to participate in epithelial cell proliferation, we examined in this study immunohistochemical localization of β-catenin, with attention to transcription activation of a target gene by β-catenin, and to evaluate nuclear accumulation β-catenin in relevance with atypical epithelium in a surface layer of oral leukoplakia. Nuclear localization of β-catenin is correlated with cyclin D1 expression in oral leukoplakia, and also Wnt3 expression in neighboring cells, known as a typical member of the Wnt family that activates β-catenin-mediated signaling [18,19], suggesting the involvement of Wnt/β-catenin signaling in the progression of dysplasia.

Results

Immunohistochemical localization of β-catenin

The expression patterns of β-catenin in oral mucosa are summarized in Table 1. In the normal oral epithelium, nuclear localization of β-catenin was not detected in all 6 samples examined; 5 out of 6 samples showed expression signals only in the cell membrane, and one sample showed cytoplasmic expression in addition to signals in the cell membrane. In the oral leukoplakia without dysplasia, 7 out of 17 samples (41%) showed expression signals in the cell membrane, and 5 samples (29%) showed expression signals in the nuclei. Oral leukoplakia with dysplasia did not show expression signals in the cell membrane, and nuclear expression was shown in 11 out of 12 samples (92%).

Localization of β-catenin in the epithelial cell membranes was observed in normal oral epithelium and oral leukoplakia, whereas expression in OSCC was low or totally absent in the cell membrane (Fig. 1). The expression of β-catenin in normal oral epithelium was observed on the cell membrane, but not within the nuclei of basal and spinous layer (Fig. 1b, 1c). In oral leukoplakia without dysplasia, the expression of β-catenin was observed on the cell membrane or both cell membrane and cytoplasm in 24% of basal and spinous layer cells (Fig. 1e, 1f). In oral leukoplakia with mild dysplasia, the expression of β-catenin was observed in the nuclei at about 30% (Fig. 1h, 1i). Oral leukoplakia with dysplasia that was especially characterized by an increased nuclear-cytoplasmic ratio, an increased number of mitotic figures, including abnormal mitoses, nuclear hyperchromatism showed nuclear expression pattern (Fig. 1k, 1l), and the expression of β-catenin in nuclei was shown in more than 80% of epithelial cells. The mean percentage of nuclear staining with β-catenin in oral leukoplakia is presented in Table 2. The nuclear expression of β-catenin in epithelial dysplasia increased depending on the grade of dysplasia, and there were significantly different between β-catenin staining without dysplasia and that with mild dysplasia ($P < 0.01$), and also between β-catenin staining with mild dysplasia and that with severe dysplasia ($P < 0.05$). The tumor parenchymal cells of OSCC (Fig. 1m-o) also showed nuclear expression pattern in 10 out of 15 specimens (67%). Incidence of nuclear localization of β-catenin was significantly different between dysplasia and normal oral epithelium ($P < 0.01$), and also between oral leukoplakia with dysplasia and those without dysplasia ($P < 0.01$).

Table 1: Expression patterns of β-catenin in oral mucosa

Tissue	Total number	Cytoplasm	Nucleus	Membrane
Normal oral epithelium	6	1	0	5
Oral leukoplakia				
Without dysplasia	17	5	5	7
With dysplasia	12	1	11*	0
Oral squamous cell carcinoma	15	5	10	0

*$P < 0.01$ between normal oral epithelium and oral leukoplakia without dysplasia by Fisher's exact test.

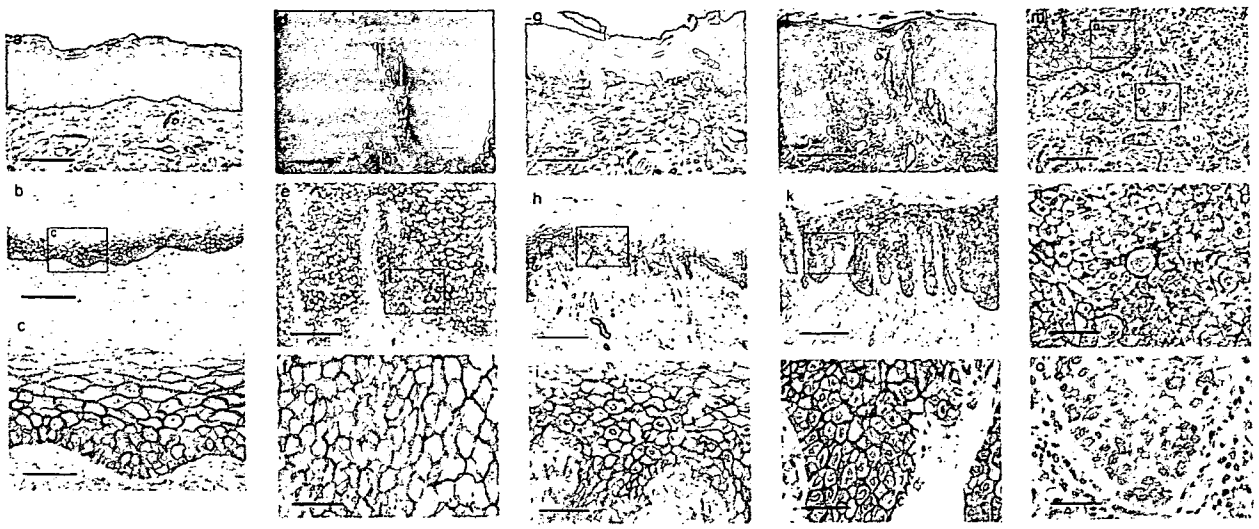

Figure 1
Immunohistochemical localization of β-catenin in normal oral epithelium (a-c), oral leukoplakia without dysplasia (d-f), oral leukoplakia with mild dysplasia (g-i), oral leukoplakia with severe dysplasia (j-l), and oral squamous cell carcinoma (m-o). (a, d, g, j) Hematoxylin and eosin staining. (b, c, e, f, h, i, k, l, m-o) β-Catenin staining. (b, c) Signals were detected in the cell membrane of the basal and spinous layer, but not in the cytoplasm and nuclei. (e, f) Signals were detected in the cell membranes and cytoplasm. (h, i) Signals were detected in the cell membranes and nuclei. (j) The area for dysplasia is characterized by an increased nuclear-cytoplasmic ratio, an increased number of mitotic figures, including abnormal mitoses, nuclear hyperchromatism. (k, l) Signals were detected in the cell membranes and nuclei. (m-o) Signals were detected in the nucleus of the epithelial dysplastic cells (n) and carcinoma cells (o) in OSCC, but cell membranous expression was weak or absent. Scale bars: (a, b, d, e, g, h, j, k, m) 200 μm; (c, f, i, l, n, o) 50 μm.

Relationship between localization of β-catenin and Wnt3 expression

We examined the expression pattern of Wnt3 in oral leukoplakia. Wnt3 was not expressed in the normal oral epithelium (data not shown). Figure 2 shows comparison of immunohistochemical staining for β-catenin, Wnt3, cyclin D1, and c-myc in oral leukoplakia without dysplasia (a-d) and those with dysplasia (e-l). Wnt3 was expressed on the epithelial cell membrane or cytoplasm where nuclear expression of β-catenin is evident, in contrast to those without nuclear expression of β-catenin (Fig. 2b, 2f, 2j). In the samples with nuclear expression of β-catenin, Wnt3 expression was shown in 13 out of 16 samples (81%), and there is significant positive correlation between the nuclear expression of β-catenin and Wnt3 staining ($P < 0.01$) (Table 3).

Table 2: Mean percentage of nuclear β-catenin staining in oral leukoplakia with and without dysplasia

Pathological diagnosis	Total number	Positive ratio (mean value)
No dysplasia	17	6.7
Mild dysplasia	9	26.9*
Severe dysplasia	3	59.7**

*$P < 0.01$ between no dysplasia samples and mild dysplasia samples by Student's *t*-test.
**$P < 0.05$ between mild dysplasia samples and severe dysplasia samples by Student's *t*-test.

We also examined other members of Wnt family, including Wnt1, Wnt5a, and Wnt7a, for immunohistochemical staining. No signal was detectable with antibodies against Wnt1 and Wnt7a in normal and leukoplakia epithelia, whereas Wnt5a signal was ubiquitously detectable in normal oral epithelium, leukoplakia, and in OSCC (data not shown). Thus, these Wnt members are unlikely to be involved in nuclear localization of β-catenin because of the absence of correlation.

Relationship with cyclin D1 and c-myc expression

Cyclin D1 was intensely expressed in the oral leukoplakia where nuclear expression of β-catenin is evident, in contrast to those without nuclear expression of β-catenin (Fig. 2). Cyclin D1 overexpression was more evident in the oral leukoplakia with dysplasia than that without dysplasia (Fig. 2c, 2g, 2k), and percentage of the cell number with positive nuclear staining is significantly different in these specimens (Fig. 3). On the other hand, the c-myc expres-

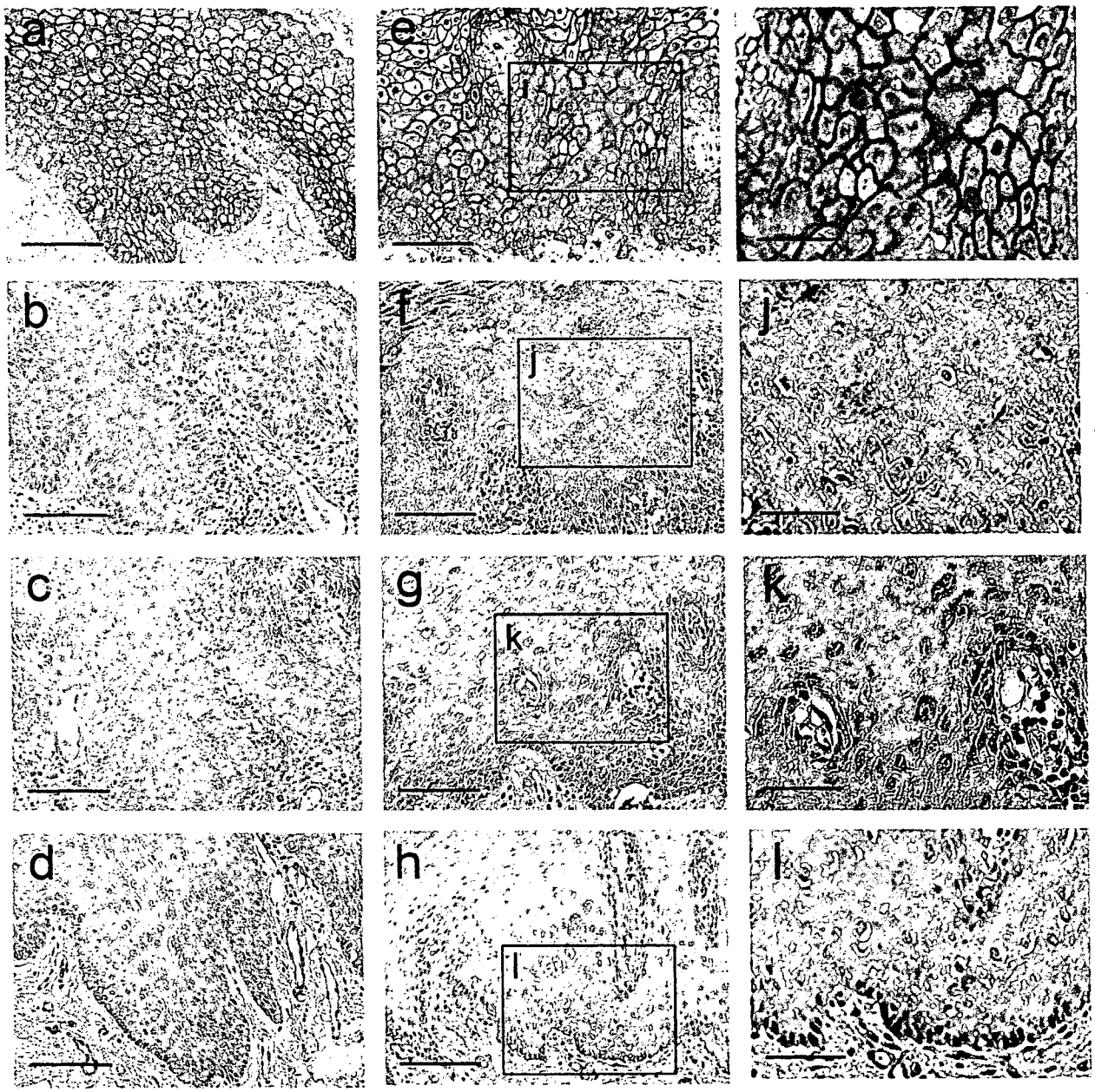

Figure 2
Comparison of immunohistochemical staining for β-catenin, Wnt3, cyclin D1, and c-myc in oral leukoplakia. (a-d) Serial sections of oral leukoplakia without dysplasia. (a) Nuclear expression of β-catenin was not observed. (b) Wnt3 expression was not observed. (c) Cyclin D1 shows weak expression. (d) Nuclear expression of c-myc is observed in the basal layer cells. (e-h) Serial sections of oral leukoplakia with dysplasia. (e, i) β-Catenin is expressed in the nuclei. (f, j) Wnt3 expression is observed on the epithelial cell membrane and in the cytoplasm. (g, k) Cyclin D1 is expressed in several epithelial cells. (h, l) c-Myc shows similar expression pattern as oral leukoplakia without dysplasia. Scale bars: (a-h) 100 μm; (i-l) 50 μm.

Table 3: Relationship between nuclear expression of β-catenin and Wnt3 expression in oral leukoplakia

β-Catenin	Total number	Wnt3	
		-	+
Nuclear expression negative	13	10	3 (23.1%)
Nuclear expression positive	16	3	13 (81.3%)*

*P < 0.01 between nuclear expression negative and positive samples by Mann-Whitney *U*-test.

sion in nuclei was not significantly different between these two specimens (Fig. 2d, 2h, 2l), suggesting independent activation of c-myc.

Discussion

β-Catenin has been identified to be a constituent of cell adhesion apparatus bound to cadherin family [20], and plays an important role for cellular movement and adhesion, as well as a signaling factor involved in canonical Wnt pathway [9]. Nuclear localization of β-catenin is known to associate with malignant transformation of colorectal cancer and other tumor [5-8]. Because cytoplasmic accumulation and subsequent nuclear translocation of β-catenin is known to be resulted from activation of canonical Wnt signaling pathways [10], subcellular localization of β-catenin is a useful marker to detect cellular conditions to proliferate actively. We compared here immunohistochemical localization of β-catenin in the normal oral epithelia, oral leukoplakia, and OSCC, aimed at elucidating relationship between proliferative activity and subcellular localization of the products.

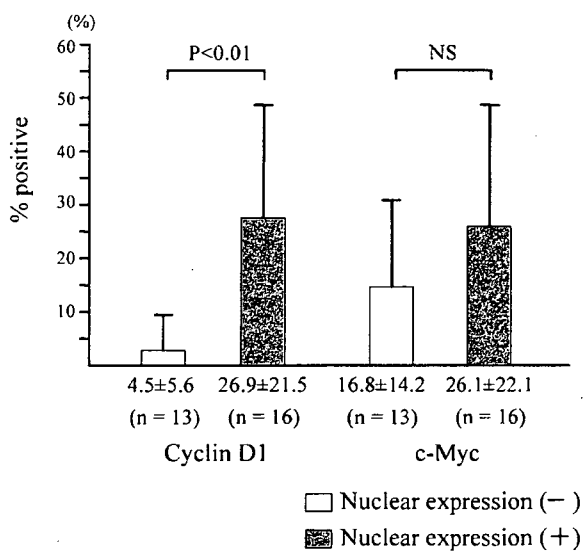

Figure 3
Relationship between subcellular localization of β-catenin and expression patterns of cyclin D1 and c-myc in oral leukoplakia. Shown by means with standard deviations.

In contrast to membranous and cytoplasmic expression, nuclear expression of β-catenin is implicated in tumor progression, but the significance of β-catenin localization in the oral epithelial dysplasia and OSCC has not yet been examined in details. The nuclear expression of β-catenin increased during progression of the severity in oral leukoplakia. Furthermore, because localization of β-catenin in normal oral epithelium and dysplasia showed significant difference, suggesting nuclear expression of β-catenin as aberrant condition. In addition, nuclear expression of β-catenin was also observed in 10 out of 15 samples of OSCC, and membranous β-catenin expression was low or totally absent in 7 out of 15 samples of OSCC (data not shown). In various malignant tumors, reduced membranous β-catenin expression was associated with the emergence of invasion or metastasis [21-24]. Our results suggest that β-catenin function in cell-cell adhesion and translocation in the nucleus are related to proliferation and invasion of OSCC.

c-Myc and cyclin D1 have been identified as target genes of the Wnt/β-catenin pathway [14-17]. Tetsu and McCormick [25] reported that expression of cyclin D1 is strongly dependent on β-catenin/TCF and has a direct effect on cell proliferation in colon carcinoma cells. Furthermore, Kovesi and Szende [26] reported that expression of cyclin D1 increased during progression of the severity in oral leukoplakia. Our results showed elevated cyclin D1 expression in the epithelial dysplasia. In oral epithelium, cyclin D1 may participate in malignant transformation. c-Myc expression was elevated in dysplasia with nuclear expression of β-catenin than that without nuclear expression, although the difference in c-myc expression is not evident.

Our studies show the aberrant Wnt signaling pathway in the oral epithelial dysplasia. Wnt3, a typical member of the Wnt family that activates β-catenin mediated signaling [18,19], was not expressed in the normal oral epithelium. In contrast, Wnt3 expression was observed in the examples showing nuclear expression of β-catenin. Both the nuclear expression of β-catenin and Wnt3 expression were observed in oral leukoplakia with dysplasia, and therefore the aberrant Wnt signaling pathway may promote malignant transformation by triggering cyclin D1 expression and consequently uncontrolled progression into the cell cycle.

These lines of evidence suggest that nuclear accumulation of β-catenin plays an important role during malignant transition of oral leukoplakia through dysplasia. In addi-

tion to pathological examination of hematoxylin and eosin-stained sections for oral leukoplakia, immunochemical staining for β-catenin is useful for diagnosis of epithelial dysplasia and precancerous changes. Evaluation of other constituents for Wnt signaling, including GSK-3, CDK, Frizzled, and LRP5/6, helps us for determination and prognosis of precancerous change of dysplasia.

Conclusion

As for the transcriptional activation of a target gene by β-catenin, elevated expression was detected in a squamous cell carcinoma and epithelial dysplasia of oral leukoplakia. Thus, Wnt/β-catenin pathway is considered to be involved in the progression of dysplasia in oral leukoplakia, as shown by nuclear expression of β-catenin and other components, including Wnt3 and cyclin D1.

Methods

Tissue samples

Formalin-fixed, paraffin-embedded samples of normal oral mucosa (6 examples), of oral squamous cell carcinoma (15 examples), and of oral leukoplakia (29 examples) were used. A fresh tissue of the oral cavity is also obtained at the time of biopsy or at oral surgery during treatment of cancerous disease after obtaining informed consent to the patients, and fixed in formalin before paraffin embedding. A histopathological diagnosis of each sample was performed by pathological staffs in the Department of Pathology in Kawasaki Medical School Hospital. According to the WHO diagnostic criteria for histological typing of cancer and pre-cancer of the oral mucosa [1], 12 examples from oral leukoplakia were diagnosed as epithelial dysplasia lesion (dysplasia). Dysplasia was diagnosed and graded as mild dysplasia (9 examples) and severe dysplasia (3 examples).

Immunohistochemistry

Sections of 4 μm thickness in paraffin embedding tissue were treated with microwave for 10 minutes in 10 mM citrate buffer (pH 6) after de-paraffin processing, followed by washing with PBS twice for 10 minutes. To remove endogenous peroxidase activity, sections were treated in 3% hydrogen peroxide solution for 15 minutes, and subsequently in PBS twice for five minutes. The primary antibodies against β-catenin (Sigma-Aldrich), Wnt3 (Santa Cruz Biotechnology), cyclin D1 (Zymed Laboratories), and c-myc (Santa Cruz Biotechnology) were used at 1/400, 1/100, 1/50, and 1/50 dilution, respectively, in PBS by incubating at room temperature for 60 min. After washing with PBS, the sections were treated with biotinylated secondary antibody for 10 min and then with peroxidase-labeled streptavidin for 10 min (LSAB PLUS kit, DAKO, Japan), and the color was developed with 3,3'-diaminobenzidine tetrahydrochloride (LSAB PLUS kit, DAKO, Japan), followed by counter staining with Mayer's hematoxylin. As a negative control, primary antibodies were omitted and the sections were incubated with the secondary antibody alone.

Evaluation of staining

For counting cells with nuclear and/or cytoplasm staining, three microscopic fields with 200-fold magnification were randomly chosen and the mean above 10% of the total cell numbers was deduced to be positive.

Evaluation of staining was carried out based on whether nuclear or cytoplasmic staining is detectable. Under microscopic field of 200-fold magnification, tissues are positive for β-catenin, if more than 10% of cells show cytoplasmic or nuclear staining. Randomly selected field in the tumor parenchymal region was used for determination of β-catenin positive cells in squamous cell carcinoma. Because signal intensity and distribution were different depending on the places in leukoplakia without dysplasia and in the normal oral epithelium, three fields were randomly selected to determine average ratio of a positive cell. In the Wnt3 staining, the cell membranous or cytoplasmic staining in the basal and spinous layer was regarded as positive. For cyclin D1 and c-myc, nuclear staining was regarded as positive. The cyclin D1 and c-myc staining was evaluated by counting at 200-fold magnification and calculating the percentage of positive cells. Statistical significance was estimated using Fisher's exact test, Mann-Whitney *U*-test, and Student's *t*-test.

Abbreviations

GSK-3 – glycogen synthase kinase-3; LEF1 – lymphoid enhancer binding factor 1; LRP – low-density lipoprotein receptor-related protein; OSCC – oral squamous cell carcinoma; PBS – phosphate-buffered saline; TCF – T cell factor.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

KI and SI carried out immunohistochemical analysis and evaluation of staining, KI also drafted the manuscript, NW participated in evaluation of immunohistochemical staining and helped to draft the manuscript, HD and TH participated to collect specimens and performed in the statistical analysis, MH and TN participated in the design and coordination of the study, TN also conceived of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

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References

1. Pindborg JJ, Reichart PA, Smith CJ, van der Waal I: **World Health Organization: histological typing of cancer and precancer of the oral mucosa.** Berlin: Springer; 1997:25-26.
2. Silverman S Jr, Gorsky M, Lozada F: **Oral leukoplakia and malignant transformation: A follow-up study of 257 patients.** *Cancer* 1984, **53**:563-568.
3. WHO Collaborating Center for Oral Precancerous Lesions: **Definition of leukoplakia and related lesions: An aid to studies on oral precancer.** *Oral Surg Oral Med Oral Pathol* 1978, **46**:518-539.
4. Reibel J: **Prognosis of oral pre-malignant lesion: significance of clinical, histopathological, and molecular biological characteristics.** *Crit Rev Oral Biol Med* 2003, **14**:47-62.
5. Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW: **Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC.** *Science* 1997, **275**:1787-1790.
6. Ogasawara N, Tsukamoto T, Mizoshita T, Inada K, Cao X, Takenaka Y, Joh T, Tatematsu M: **Mutations and nuclear accumulation of beta-catenin correlate with intestinal phenotypic expression in human gastric cancer.** *Histopathology* 2006, **49**:612-621.
7. Takayama T, Shiozaki H, Shibamoto S, Oka H, Kimura Y, Tamura S, Inoue M, Monden T, Ito F, Monden M: **Beta-catenin expression in human cancers.** *Am J Pathol* 1996, **148**:39-46.
8. Zhou XB, Lu N, Zhang W, Quan LP, Lin DM, Wang QH, Li YL, Xu NZ: **Expression and significance of beta-catenin in esophageal carcinoma.** *Ai Zheng* 2002, **21**:877-880.
9. Li H, Pamukcu R, Thompson WJ: **β-Catenin signaling.** *Cancer Biol Ther* 2002, **1**:621-625.
10. Lustig B, Behrens J: **The Wnt signaling pathway and its role in tumor development.** *J Cancer Res Clin Oncol* 2003, **129**:199-221.
11. Endo Y, Rubin JS: **Wnt signaling and neurite outgrowth: Insights and questions.** *Cancer Sci* 2007, **98**:1311-1317.
12. Behrens J, von Kries JP, Kühl M, Bruhn L, Wedlich D, Grosschedl R, Birchmeier W: **Functional interaction of beta-catenin with the transcription factor LEF-1.** *Nature* 1996, **382**:638-642.
13. Huber O, Korn R, McLaughlin J, Ohsugi M, Herrmann BG, Kemler R: **Nuclear localization of beta-catenin by interaction with transcription factor LEF-1.** *Mech Dev* 1996, **59**:3-10.
14. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW: **Identification of c-MYC as a target of the APC pathway.** *Science* 1998, **281**:1509-1512.
15. Bettess MD, Dubois N, Murphy MJ, Dubey C, Roger C, Robine S, Trumpp A: **c-Myc is required for the formation of intestinal crypts but dispensable for homeostasis of the adult intestinal epithelium.** *Mol Cell Biol* 2005, **25**:7868-7878.
16. Sherr CJ: **Cancer cell cycles.** *Science* 1996, **274**:1672-1677.
17. Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben-Ze'ev A: **The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway.** *Proc Natl Acad Sci USA* 1999, **96**:5522-5527.
18. Filali M, Cheng N, Abbott D, Leontiev V, Engelhardt JF: **Wnt-3A/β-catenin signaling induces transcription from the LEF-1 promotor.** *J Biol Chem* 2002, **277**:33398-33410.
19. Uraguchi M, Morikawa M, Shirakawa M, Sanada K, Imai K: **Activation of WNT family expression and signaling in squamous cell carcinomas of the oral cavity.** *J Dent Res* 2004, **83**:327-332.
20. Yap AS, Brieher WM, Gumbiner BM: **Molecular and functional analysis of cadherin-based adherens junctions.** *Annu Rev Cell Dev Biol* 1997, **13**:119-146.
21. Bankfalvi A, Terpe HJ, Breukelmann D, Bier B, Rempe D, Pschadka G, Krech R, Lelle RJ, Boecker W: **Immunophenotypic and prognostic analysis of E-cadherin and β-catenin expression during breast carcinogenesis and tumour progression: a comparative study with CD44.** *Histopathology* 1999, **34**:25-34.
22. Karayiannakis AJ, Nakopoulou L, Gakiopoulou H, Keramopoulos A, Davaris PS, Pignatelli M: **Expression patterns of β-catenin in in situ and invasive breast cancer.** *Eur J Surg Oncol* 2001, **27**:31-36.
23. Pirinen RT, Hirvikoski P, Johansson RT, Hollmen S, Kosma VM: **Reduced expression of α-catenin, β-catenin, and γ-catenin is associated with high cell proliferative activity and poor differentiation in non-small cell lung cancer.** *J Clin Pathol* 2001, **54**:391-395.
24. Lo Muzio L, Staibano S, Pannone G, Grieco M, Mignogna MD, Cerrato A, Testa NF, De Rosa G: **Beta- and Gamma-catenin expression in oral squamaous cell carcinomas.** *Anticancer Res* 1999, **19**:3817-3826.
25. Tetsu O, McCormick F: **β-Catenin regulates expression of cyclin D1 in colon carcinoma cells.** *Nature* 1999, **398**:422-426.
26. Kovesi G, Szende B: **Prognostic value of cyclin D1, p27, and p63 in oral leukoplakia.** *J Oral Pathol Med* 2006, **35**:274-277.

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Transgenic expression of a myostatin inhibitor derived from follistatin increases skeletal muscle mass and ameliorates dystrophic pathology in *mdx* mice

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ABSTRACT **Myostatin is a potent negative regulator of skeletal muscle growth. Therefore, myostatin inhibition offers a novel therapeutic strategy for muscular dystrophy by restoring skeletal muscle mass and suppressing the progression of muscle degeneration. The known myostatin inhibitors include myostatin propeptide, follistatin, follistatin-related proteins, and myostatin antibodies. Although follistatin shows potent myostatin-inhibiting activities, it also acts as an efficient inhibitor of activins. Because activins are involved in multiple functions in various organs, their blockade by follistatin would affect multiple tissues other than skeletal muscles. In the present study, we report the characterization of a myostatin inhibitor derived from follistatin, which does not affect activin signaling. The dissociation constants (K_d) of follistatin to activin and myostatin are 1.72 nM and 12.3 nM, respectively. By contrast, the dissociation constants (K_d) of a follistatin-derived myostatin inhibitor, designated FS I-I, to activin and myostatin are 64.3 μM and 46.8 nM, respectively. Transgenic mice expressing FS I-I, under the control of a skeletal muscle-specific promoter showed increased skeletal muscle mass and strength. Hyperplasia and hypertrophy were both observed. We crossed FS I-I transgenic mice with *mdx* mice, a model for Duchenne muscular dystrophy. Notably, the skeletal muscles in the *mdx*/FS I-I mice showed enlargement and reduced cell infiltration. Muscle strength is also recovered in the *mdx*/FS I-I mice. These results indicate that myostatin blockade by FS I-I has a therapeutic potential for muscular dystrophy.—Nakatani, M., Takehara, Y., Sugino, H., Matsumoto, M., Hashimoto, O., Hasegawa, Y., Murakami, T., Uezumi, A., Takeda, S., Noji, S., Sunada, Y., Tsuchida, K. Transgenic expression of a myostatin inhibitor derived from follistatin increases skeletal muscle mass and ameliorates dystrophic pathology in *mdx* mice. *FASEB J.* 22, 477–487 (2008)**

Key Words: Duchenne muscular dystrophy · activin · therapy · myostatin blockade

MYOSTATIN, ALSO KNOWN AS GROWTH and differentiation factor 8 (GDF8), belongs to the transforming growth factor (TGF)-β superfamily and regulates skeletal muscle mass (1, 2). Mice, cattle, sheep, and humans with genetic mutations in the myostatin gene show marked increases in skeletal muscle mass, due to hypertrophy and/or hyperplasia (3–6). Therefore, myostatin is a rational candidate for a muscle chalone, defined as a molecule that regulates the size of a particular tissue (1). Myostatin is predominantly expressed in skeletal muscles and to a lesser extent in adipose tissues, and circulates in the serum. Intriguingly, myostatin forms a complex with a number of other proteins, such as myostatin propeptide and follistatin-related gene product (FLRG) (7).

Interestingly, inhibition of myostatin activity is capable of increasing muscle mass and strength in the postnatal period and even in adults. These observations suggest that targeting of myostatin would be suitable as a therapy for degenerating muscle diseases, such as muscular dystrophy and cachexia, as well as for preventing muscle wasting due to aging (8–10). In fact, antibody-mediated myostatin blockade in *mdx* mice, a model for Duchenne muscular dystrophy, was found to ameliorate the pathophysiology and muscle weakness (11). Myostatin propeptide-mediated amelioration of the symptoms in *mdx* mice has also been reported (12).

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