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Long-Term In Vitro Analysis of Limb Cartilage Development: Involvement of Wnt Signaling

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Abstract Endochondral skeletal development involves the condensation of mesenchymal cells, their differentiation into chondrocytes, followed by chondrocyte maturation, hypertrophy, and matrix mineralization, and replacement by osteoblasts. The Wnt family of secreted proteins have been shown to play important roles in vertebrate limb formation. To examine the role(s) of Wnt members and their transmembrane-spanning receptor(s), Frizzled (fz), we retrovirally misexpressed *Wnt-5a*, *Wnt-7a*, chicken *frizzled-1* (Chfz-1), and *frizzled-7* (Chfz-7) in long-term (21 day) high density, micromass cultures of stage 23/24 chick embryonic limb mesenchyme. This culture system recapitulates in vitro the entire differentiation (days 1–10), growth (days 5–12), and maturation and hypertrophy (from day 12 on) program of cartilage development. Wnt-7a misexpression severely inhibited chondrogenesis from day 7 onward. Wnt-5a misexpression resulted in a poor hypertrophic phenotype by day 14. Chfz-7 misexpression caused a slight delay of chondrocyte maturation based on histology, whereas Chfz-1 misexpression did not affect the chondrogenic phenotype. Misexpression of all Wnt members decreased collagen type X expression and alkaline phosphatase activity at day 21. Our findings implicate functional role(s) for Wnt signaling throughout embryonic cartilage development, and show the utility of the long-term in vitro limb mesenchyme culture system for such studies. J. Cell. Biochem. 93: 526–541, 2004.
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Long bones of the vertebrate limb develop via endochondral ossification, a process that involves the condensation of loosely packed mesenchymal cells, the differentiation of these cells into chondrocytes, and a period of hypertrophy during which the tissue becomes vascularized, mineralized, and replaced by osteoblasts. Embryonic cartilage development is a highly regulated and complex process, and is controlled by a number of factors. These include members of the transforming growth factor-β (TGF-β) superfamily, the fibroblast growth factor (FGF) family, retinoids, hedgehog gene products, parathyroid hormone-related peptide (PTHrP), cell adhesion molecules (e.g., N-CAM and cadherins), and extracellular matrix components [see reviews by DeLise et al., 2000; Shum et al., 2003].

Recently, the Wnt family of secreted proteins has been shown to play important functional roles in vertebrate development, including involvement in the normal formation of the limbs [Nusse and Varmus, 1992; Wodarz and Nusse, 1998; Yang et al., 2003]. Wnts are comprised of at least 15 secreted proteins that have been shown to associate with the cell membrane and extracellular matrix in target tissues. Through interaction with their receptor, Frizzled, as well as other co-receptors [Oishi

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et al., 2003; Tolwinski et al., 2003], Wnts are thought to mediate several effectors in a localized signaling cascade resulting in the regulation of cell adhesion and/or gene expression. This canonical pathway occurs through the modulation of β-catenin and the activity of the lymphocyte enhancing factor (LEF)/T-cell factor (TCF) family of transcription factors [Nusse and Varmus, 1992; Cadigan and Nusse, 1997], while recent studies have described alternative signaling pathways, involving G-protein-like [Slusarski et al., 1997a; Veeman et al., 2003] components and a number of downstream signaling cascades [Slusarski et al., 1997b; Scheldahl et al., 1999; Veeman et al., 2003]. Members of the Wnt family have been implicated in embryonic pattern formation as well as the morphogenesis of various structures during both vertebrate and invertebrate development [Dickinson and McMahon, 1992; Nusse and Varmus, 1992; Church and Francis-West, 2002].

Wnt-5a and Wnt-7a in particular are expressed in a spatio-temporally, specific pattern within chick embryonic limb buds [Dealy et al., 1993]. Wnt-5a expression follows a proximodistal gradient in the limb mesoderm and is also found in the apical ectodermal ridge (AER), while Wnt-7a is expressed exclusively within the dorsal ectoderm. These patterns suggest roles for these Wnts in proximodistal and dorsoventral patterning, as well as a role for Wnt-5a in the maintenance of limb outgrowth [Yang, 2003]. Recent work has shown complementary activity of different Wnts during chondrogenesis and limb development [Yang et al., 2003]. Our recent study [Tufan and Tuan, 2001] reveals the inhibitory effects of Wnt-7a misexpression during chick chondrogenesis in vitro, and that it may be due to an alteration of N-cadherin-related activities. Cells misexpressing Wnt-5a appeared to proceed normally though condensation and differentiation in this system. However, two in vivo studies have demonstrated that ectopic expression of Wnt-5a delays chondrocyte maturation and collagen type X expression, late events of limb cartilage development [Kawakami et al., 1999; Hartmann and Tabin, 2000].

More recently, the involvement of the Wnt receptor, Frizzled, in limb cartilage development has been investigated. *frizzled* encodes a seven transmembrane-spanning protein which includes a cysteine-rich extracellular ligand-binding domain that has been shown to interact with some Wnts [Vinson et al., 1989; Bhanot et al., 1996]. So far there have been more than 10 vertebrate *frizzled* genes identified, some of which are expressed in the developing limb. In the chick embryo, as the limb buds emerge at stage 17, several Frizzled (Chfz) proteins, including Chfz-1 and Chfz-7, exhibit a relatively uniform distribution in the ectoderm and mesenchyme [Kengaku et al., 1997]. As the limb develops, Chfz-1 becomes restricted to the ventral ectoderm and mesenchyme, while Chfz-7 is expressed along the proximodistal axis with the highest expression in the distal mesoderm beneath the AER [Kengaku et al., 1997]. We recently demonstrated that misexpression of Chfz-7 inhibits the cellular condensation step required for chondrogenesis [Tufan et al., 2002]. Hartmann and Tabin [2000] have reported that the misexpression of Chfz-1 and Chfz-7 results in severe shortening of cartilage elements in vivo. However, the binding specificity of these receptors and their exact functional roles during chondrogenesis are presently not known.

Thus, while members of both Wnt and Frizzled families are implicated to play significant roles in cartilage development, only the early stage of cellular condensation and chondrogenic differentiation has been analyzed in cell cultures in vitro. In this study, we have applied long-term, high-density cultures of embryonic limb mesenchymal cells, a system that recapitulates the entire program of cartilage development, including condensation, differentiation, maturation, and hypertrophy [Mello and Tuan, 1999], to examine the role of Wnt/frizzled signaling. We used retroviral constructs to overexpress Wnt-5a, Wnt-7a, Chfz-1, and Chfz-7 in long-term (21 day) chick mesenchymal cell micromass cultures. We found that retrovirally mediated overexpression of Wnt-7a completely halted the processes of chondrocyte differentiation and maturation, while misexpression of Wnt-5a, Chfz-1, or Chfz-7 delayed post-differentiation events to varying degrees.

MATERIALS AND METHODS

Chicken Embryos

Fertilized SPF-11 (C/E) White Leghorn chicken eggs were obtained from Charles River SPAFAS, Inc. (Prestion, CT) and incubated at 37.5°C in a humidified egg incubator for

4 days (Hamburger–Hamilton stage 23/24) [Hamburger and Hamilton, 1992].

Micromass Culture and Electroporation-Mediated Retroviral Transfection of Embryonic Chick Limb Bud Mesenchymal Cells

Chick limb bud mesenchymal cells were isolated and established as micromass cultures as described previously [Ahrens et al., 1977; San Antonio and Tuan, 1986]. Before plating, cells were adjusted to a concentration of 40×10^6 cells/ml and transfected with retroviral (RCAS) constructs, containing empty RCAS vector, RCAS–Wnt-5a [Kawakami et al., 1999], RCAS–Wnt-7a [Yang and Niswander, 1995], RCAS–Chfz-1 [Hartmann and Tabin, 2000], or RCAS–Chfz-7 [Hartmann and Tabin, 2000], by means of an electroporation method described by DeLise and Tuan [2000] which was used in several of our recent studies [e.g., Tufan and Tuan, 2001; Delise and Tuan, 2002; Tufan et al., 2002; Coleman and Tuan, 2003]. Cultures were plated in Ham's F12 medium (Gibco-BRL, Gaithersburg, MD) containing 1 mM $CaCl_2$, 10,000 U/ml penicillin–streptomycin (Sigma Chemical Company, St. Louis, MO), 1% glucose, and 10% fetal bovine serum (FBS; HyClone, Logan, UT). From day 2 on, the medium was replaced with a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (DMEM/F12; Gibco) supplemented with 2.5 mM β-glycerophosphate, 0.3 mg glutamine/ml, and 25 mg ascorbate/ml as well as $CaCl_2$, antibiotics, glucose, and FBS as described by Mello and Tuan [1999].

Morphology and Histology of Cultured Cells

By day 7, large nodules formed over most cultures and obstructed observation by phase-contrast light microscopy. Therefore, cultures maintained for 7 days or longer were examined after paraffin-embedding and sectioning. Cultures were fixed in 4% paraformaldehyde, dehydrated, embedded in Paraplast-X (Oxford Labware, St. Louis, MO), and sectioned at 10 μm thickness. Sections from cultures harvested on days 7, 14, and 21 were stained with hematoxylin and eosin (H/E), Alcian Blue (pH 1.0) for sulfated proteoglycans found in cartilage, and/or Direct Red (1% aqueous solution; pH 5–6) to detect matrix mineralization. Whole amount cultures were also fixed in 4% paraformaldehyde and stained with Alcian Blue or hematoxylin to examine gross morphology.

Scanning Electron Microscopy (SEM)

At day 14, micromass cultures were fixed in glutaraldehyde, post-fixed in 1% osmonium tetroxide, and then critical point dried. Specimens were sputter-coated with gold and viewed using a JEOL 35-C scanning electron microscope.

Cell Proliferation

5-Bromo-2′-deoxyuridine (BrdU) Labeling and Detection Kit II (Roche Molecular Biochemicals, Mannheim, Germany) were used according to the manufacturer's protocol in order to detect sites of cell proliferation. Briefly, cells were incubated in medium containing 10 μM BrdU for approximately 1 h at 37°C and 5% CO_2, fixed with 4% paraformaldehyde, and sectioned as described above. Cells were processed for immunohistochemistry to detect BrdU incorporation using anti-BrdU antibodies and alkaline phosphatase conjugated secondary antibodies as supplied by the manufacturer. In order to block endogenous alkaline phosphatase activity, 1 mM levamisole was added to the developer solution. Positive cells were then visualized by light microscopy. A negative control (no primary antibody) was included to detect nonspecific background staining.

Hypertrophy

Given the round shape of the cells, mean cell area (±SE) of each culture at each time point was estimated using IP Lab software, version 3.2. (Scanalytics, Fairfax, VA). Cells were measured by calculating the area of the best fitting elliptical frame drawn around the cell membrane ($n > 100$ for each set of measurements; 3 sets of measurements per group). Groups were compared at days 14 and 21 to the corresponding group at day 7, and each transfected group was compared to control at each time point. Statistical analysis was performed using ANOVA and Scheffe's F-test, and significance considered at $P \leq 0.05$.

Alkaline Phosphatase Activity

Histochemical detection of alkaline phosphatase enzyme activity was performed according to the method of Stott and Chuong [1997] with some modification. Briefly, paraffin-embedded sections were cleared, rehydrated, and incubated in a Tris-Mg buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 10 min.

The sections were then incubated in Tris-Mg buffer containing 225 μg/ml NBT and 175 μg/ml BCIP (Zymed, South San Francisco, CA). Color was developed overnight at room temperature.

Antibodies

X-AC9, a monoclonal antibody to chicken collagen type X, was purchased from Developmental Studies Hybridoma Bank (University of Iowa). Monoclonal antibody against β-catenin was purchased from Transduction Laboratories (#610154; San Diego, CA), and monoclonal antibody against N-cadherin was purchased from Zymed (#18-0224).

Protein Isolation and Immunoblotting

Cultures were washed two times in ice-cold PBS. Proteins were extracted at 4°C with ice-cold RIPA buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% IGEPAL, 0.1% SDS, 0.1 Na deoxycholate, pH 7.4) containing protease and phosphatase inhibitors added just before use (1 mM PMSF, 2 μg/ml aprotinin, 20 μM leupeptin, 1 mM Na orthovanadate, 40 mM NaF) [Pukac et al., 1997]. In order to lyse cells, 1 ml of RIPA buffer was added per two micromass cultures, and cultures were mechanically disrupted by grinding in a disposable tissue grinder (Kendall, Mansfield, MA) and passing several times through a sterile syringe fitted with a 25G11/2 needle. Protein concentration was determined using the BCA Assay (Pierce Chemical Company, Rockford, IL). Fifteen to 20 μg of protein was fractionated by 10% SDS–PAGE and transferred electrophoretically onto 0.2 μm nitrocellulose membranes (Micron Separations, Inc., Westborough, MA). Filters were stained for 5 min with Ponceau S (Sigma) and destained for 5 min with deionized H_2O to ensure equivalent protein load. Filters were blocked for 1 h with 5% non-fat dry milk and incubated for 2 h with primary antibody (X-AC9, 0.2 μg/ml; anti-β-catenin, 0.5 μg/ml; anti-N-cadherin, 0.2 μg/ml) in PBS. Filters were washed in PBST (PBS with 0.1% Tween-20) and incubated for 1 h with alkaline phosphatase-conjugated goat anti-mouse IgG (1:3,000 in PBS; Sigma). Blots were developed at room temperature with BCIP/NBT (Zymed).

Reverse-Transcription-Polymerase Chain Reaction (RT-PCR)

Two micromass cultures were combined with 1 ml of Tri-Reagent (Sigma) and mechanically disrupted as described above. Total RNA was isolated from micromass cultures according to the manufacturer's protocol. RNA was quantified on the basis of A_{260}, and examined for integrity by agarose gel electrophoresis and ethidium bromide staining. One microgram of template RNA was processed for RT and amplified by PCR using the SuperScript One-Step RT-PCR with Platinum Taq kit (Life Technologies, Rockville, MD) according to the manufacturer's protocol. Briefly, a 50 μl aliquot of 2× Reaction Mix, 25 pM of gene-specific primers (Integrated DNA Technologies; see Table I for selected genes and primers designed), 1 μg RT/Platinum Taq mix, and 22 μl of RNase-free dH_2O were added to template RNA,

TABLE I. Primers for Reverse-Transcription-Polymerase Chain Reaction (RT-PCR) Analysis of Gene Expression

Gene	Primer sequence (5′ to 3′)	cDNA region	Product size (bp)
Wnt-5a[a]	Forward: GATTTCCGCAAGGTAGGCGATG	Identical to nt 1048–1069	
	Reverse: CACAACTTGCATTTCACGTAGCAG	Complementary to nt 1362–1385	338
Wnt-7a[b]	Forward: CAAGGTCTTCGTGGATGCCCG	Identical to nt 750–770	
	Reverse: TGGTGTGTGTTGTAGCCACGGC	Complementary to nt 1190–1211	462
Chfz-1[c]	Forward: TTCGTGGGCATCAACAACG	Identical to nt 1279–1297	
	Reverse: TGCTGTGGTTATTGGGGCAG	Complementary to nt 1587–1606	326
Chfz-7[d]	Forward: CCCAAGGCACCAAGAAAGAAG	Identical to nt 979–999	
	Reverse: TGCGAAACAAGGACACAAAGC	Complementary to nt 1324–1344	366
Collagen type X[e]	Forward: ATTGCCAGGGATGAAGGGACATAG	Identical to nt 1044–1067	
	Reverse: AGGTATTCCTGAAGGTCCTCTTGG	Complementary to nt 1459–1482	439
GAPDH[f]	Forward: AGTCATCCCTGAGCTGAATG	Identical to nt 680–699	
	Reverse: AGGATCAAGTCCACAACACG	Complementary to nt 990–1009	330

Genbank accession numbers.
[a]AB006014 [Kawakami et al., 1999].
[b]AK004683 [Carninci and Hayashizaki, 1999].
[c]AF031830 [Kengaku et al., 1997].
[d]AF031831 [Kengaku et al., 1997].
[e]M13496 [Ninomiya et al., 1986].
[f]J00849 [Dugaiczyk et al., 1983].

and RT-PCR reactions were performed in a Perkin-Elmer DNA thermal cycler under the following conditions: 1 cycle of 50°C for 30 min, 92°C for 2 min (cDNA synthesis and predenaturation); 25–40 cycles of 94°C for 30 s (denaturation), 58°C for 30 s (annealing), 72°C for 1 min (extension); and reactions were prolonged at 72°C for 10 min, terminated at 4°C, and stored at −20°C. PCR products were visualized by agarose gel electrophoresis and ethidium bromide staining.

Northern Blot Analysis

Total RNA was isolated as described above for RT-PCR and Northern blot analysis was performed as previously described by Tufan and Tuan [2001]. Random-prime ^{32}P-labeled probes (Amersham Biosciences Corp., Piscataway, NJ) used in this study included collagen type X cDNA [Ninomiya et al., 1986] and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Clontech Laboratories, Inc., Palo Alto, CA).

Apoptosis

Internucleosomal DNA degradation was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) [Gavrieli et al., 1992]. Tissue sections were incubated with terminal transferase to label the 3′-end of fragmented nuclear DNA with fluorescein-conjugated dUTP. The label was detected immunohistochemically by peroxidase-labeled antibodies against fluorescein (In situ Cell Death Detection Kit, POD, Roche Molecular Biochemicals) developed with a DAB substrate solution (Zymed). A positive control (day 3 untransfected micromass culture with DNaseI-induced strand breaks) and negative control (no primary antibody) were included to detect false positive or negative staining.

RESULTS

RCAS-Mediated Wnt and Frizzled Overexpression

RT-PCR was carried out using gene-specific primer pairs to validate RCAS-mediated overexpression of exogenous *Wnt-5a*, *Wnt-7a*, *Chfz-1*, and *Chfz-7* mRNA on days 7, 14, and 21 of culture. GAPDH mRNA expression was also assessed to normalize RNA load. High levels of *Wnt-5a*, *Wnt-7a*, *Chfz-1*, or *Chfz-7* transcripts were expressed in correspondingly transfected cells consistently through day 21 (Fig. 1; similar results seen on days 7 and 14, data not shown). Interestingly, a higher level of *Chfz-1* and *Chfz-7* transcript was also found in cultures overexpressing Wnt-7a (Fig. 1), a biological response suggesting the possibility that these two Frizzleds may be candidate receptors for Wnt-7a.

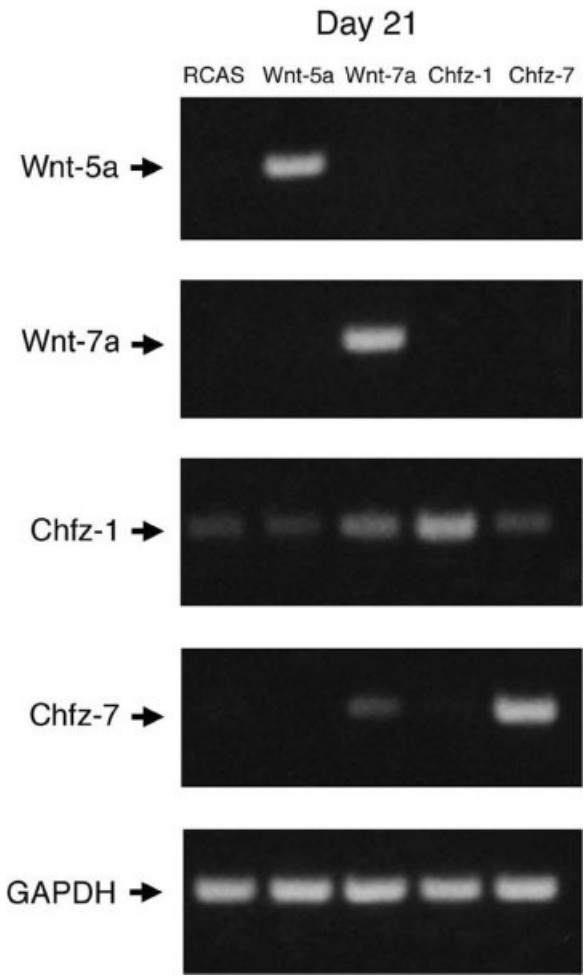

Fig. 1. Reverse-transcription-polymerase chain reaction (RT-PCR) analysis of RCAS-mediated overexpression in transfected chick limb mesenchyme micromass cultures. Total RNA was isolated from cultures on day 21; RT-PCR was performed using primers specific to *Wnt-5a, Wnt-7a, Chfz-1,* or *Chfz-7* (Table I) and products were analyzed by gel electrophoresis. Cultures expressing exogenous Wnt or Chfz clearly showed increased transcription of the respective gene.

Morphology and Histology

We examined the cultures for differences in gross and cellular morphology. As observed by phase-contrast light microscopy, cartilage nodules were present in cultures transfected with Wnt-5a, Chfz-1, or Chfz-7 by day 3. Grossly, cartilage nodules increased in size

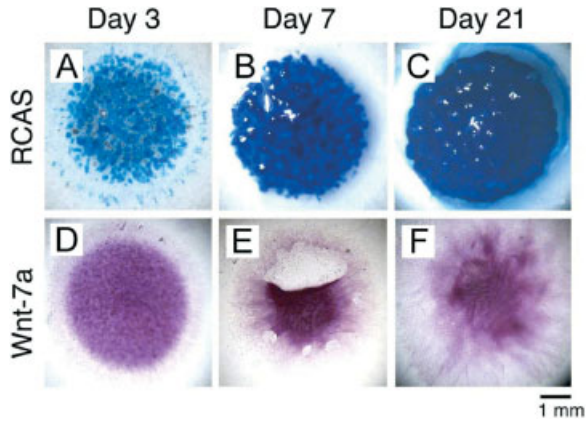

Fig. 2. Gross morphology of long-term limb mesenchyme micromass cultures. Whole-mount cultures were transfected with RCAS-empty vector as a control and stained with Alcian Blue (**A**–**C**), or transfected with RCAS–Wnt-7a and stained with hematoxylin (**D**–**F**). Control cultures exhibited the appearance of discrete cartilaginous nodules by day 3 (A), which increased in number and size through day 21 (C). Cultures transfected with RCAS–Wnt-7a did not exhibit nodular formation at any time point, and consisted largely of undifferentiated, fibroblastic cells through day 21 (F).

and number over time, such that by day 21, the entire micromass culture became a round, three-dimensional, nodular structure approximately 3–4 mm in diameter and 1–2 mm in height. These cultures stained positively with Alcian Blue (Fig. 2A–C), indicating a sulfated proteoglycan-rich matrix, a characteristic of cartilage. Cells transfected with RCAS–Wnt-

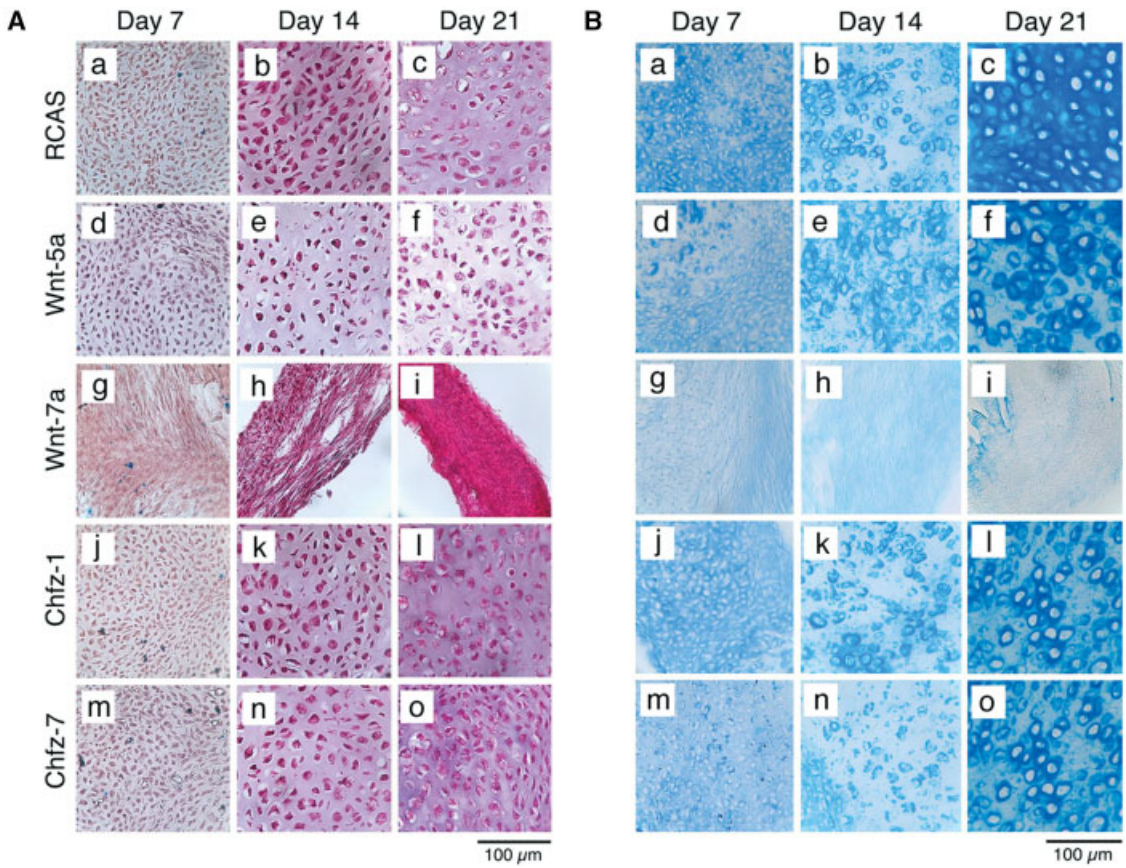

Fig. 3. Histological morphology of histological sections of control and transfected limb mesenchyme micromass cultures. Histological sections of micromass cultures on days 7, 14, and 21 were stained with hematoxylin and eosin (H/E) (**A**), or Alcian Blue (**B**). Cultures were transfected with control RCAS vector (**a**–**c**), Wnt-5a (**d**–**f**), Wnt-7a (**g**–**i**), Chfz-1 (**j**–**l**), or Chfz-7 (**m**–**o**). There was an increase in cell size and ECM production over time in all cultures except those overexpressing Wnt-7a, which consisted mainly of undifferentiated, fibroblastic cells (A, g–i). Cultures overexpressing Wnt-5a exhibited a poor hypertrophic phenotype at day 14 (A, e) compared to controls (A, b). Alcian Blue staining was apparent by day 7 (B) and became more intense over time in all cultures, except those overexpressing Wnt-7a, where chondrogenesis was strongly inhibited (B, g–i). Cultures misexpressing Wnt-5a or Chfz-7 exhibited an initial delay in maturation (B, d; B, m), but appeared phenotypically similar to control (B, c) at day 21 (B, f; B, o).

7a did not form nodules and exhibited no vertical growth; these cultures lost their circular appearance over time and the cells spread laterally across the plate (Fig. 2D–F). Cultures overexpressing Wnt-7a did not stain positively for Alcian Blue, and maintained an elongated, fibroblastic appearance, indicating that the cells did not differentiate into chondrocytes.

Light microscopy of histological sections stained with H/E (Fig. 3A) and Alcian Blue (Fig. 3B) showed that cultures transfected with RCAS alone exhibited an increase in size as well as in the amount of sulfated ECM proteoglycans from days 7 to 21 (Fig. 3A,B; a–c), typical of chondrocytes in vivo and similar to untransfected chondrocytes cultured under the same conditions [Mello and Tuan, 1999]. In contrast, misexpression of Wnt-7a halted the process of endochondral ossification before day 7, resulting in the formation of cells showing fibroblastic morphology, and not the chondrogenic phenotype normally observed by this time (Fig. 3A; g–i), and little to no positive Alcian Blue staining (Fig. 3B; g–i). Misexpression of Wnt-5a resulted in an initial delay in chondrocyte maturation around day 7 (Fig. 3B; d) as demonstrated by reduced Alcian Blue staining compared to RCAS control (Fig. 3B; a), indicating a delay in the maturation of differentiated chondrocytes. Although Alcian Blue staining of these cultures is comparable to the RCAS control by day 14 (Fig. 3B; e), H/E staining reveals a relatively poor hypertrophic phenotype at this time point (Fig. 3A; e), suggesting that recovery from a delay at day 7 is not complete by day 14. By day 21, cultures overexpressing Wnt-5a did show little less Alcian Blue staining than the controls (Fig. 3B; c, f), but appeared phenotypically similar to controls (Fig. 3A,B; c, f).

Previously, we have observed an inhibitory effect of Chfz-7 misexpression on early chondrogenic events, i.e., mesenchymal condensation [Tufan et al., 2002]. In this system, cultures misexpressing Chfz-7 were stained less with Alcian Blue at days 7, 14, and 21 compared to controls (Fig. 3B; m–o). However, by day 21, these cultures appeared phenotypically similar to control (Fig. 3B; o), and H/E staining of these cultures appeared relatively similar to controls at all time points (Fig. 3A; m–o). Taken together with our previous findings, these results suggest that overexpression of Chfz-7 may result in a delay in chondrocyte differentiation which retards but does not halt the maturation process, and is at least partially overcome by day 21. Interestingly, although a recent in vivo study has shown that overexpression of Chfz-1 in the chick embryo results in severe shortening of the limbs [Hartmann and Tabin, 2000], cultures overexpressing Chfz-1 in our system appeared phenotypically similar to controls (Fig. 3A,B; j–l) except the fact that they were little less stained with Alcian Blue, specially at day 21 (Fig. 3A; j–l). A statistically significant increase in cell area as a function of culture time could be seen in H/E stained sections for all cultures except those overexpressing Wnt-7a (Fig. 4; Wnt-7a omitted), as expected for cells proceeding through the cartilage maturation and hypertrophic phases of development. Cell area was comparable among control and treated groups at all time points except day 14, when cells overexpressing Wnt-5a were significantly smaller than control and other treated cultures, further demonstrating that Wnt-5a overexpression results in a delay in chondrocyte maturation and hypertrophy.

SEM

At day 14, whole mount control and transfected cultures were processed for SEM to examine cellular morphology (Fig. 5). Control cultures displayed discrete clusters of spheroid hypertrophic cells (Fig. 5A), while Wnt-5a overexpressing cultures exhibited a number of cartilaginous aggregates similar to controls as well as less defined, poorly hypertrophic regions (Fig. 5B). Cells overexpressing Wnt-7a were

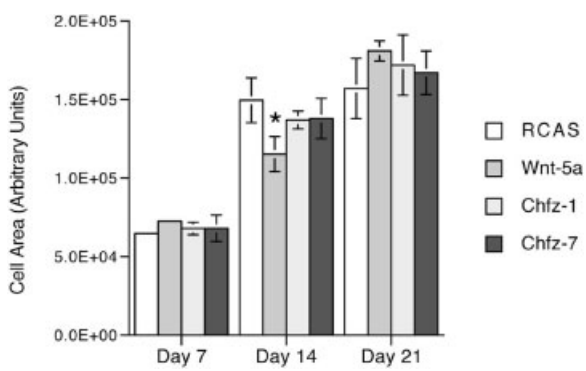

Fig. 4. Quantiation of change in cell size in long-term limb mesenchyme micromass cultures as a function of time in culture. Cell area was determined histomorphometrically as described in ''Materials and Methods'' ($n \geq 300$ cells per group). A statistically significant increase in cell area was seen over time within each culture group. Wnt-5a overexpressing cells were significantly smaller than controls at day 14 ($P < 0.05$).

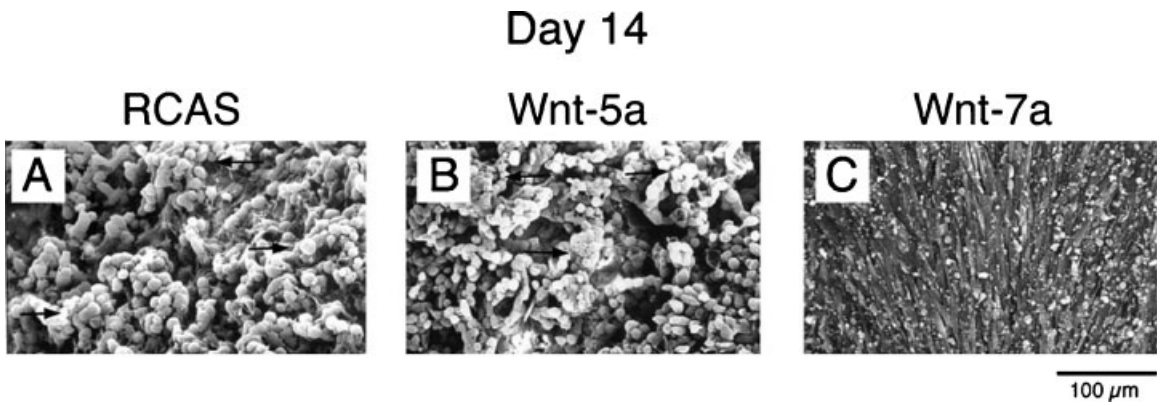

Fig. 5. Morphology of day 14 limb mesenchyme micromass cultures examined by scanning electron microscopy (SEM). Distinct cartilage nodules were visible in control cultures (**A**). Wnt-5a overexpressing cultures exhibited a mixture of hypertrophic aggregates and less round, poorly hypertrophic areas (arrows; **B**). Wnt-7a overexpressing cultures retained an undifferentiated, fibroblastic cellular phenotype (**C**). Cultures overexpressing Chfz-1 or Chfz-7 appeared similar to controls (data not shown).

highly fibroblastic and appeared to lack the chondrocyte phenotype (Fig. 5C). Cultures overexpressing Chfz-1 or Chfz-7 appeared relatively similar to controls (data not shown).

Collagen Type X Expression

We next compared levels of collagen type X, a specific marker for hypertrophic chondrocytes. In control cultures, RNA transcripts of collagen type X were detected by RT-PCR in cultures from day 14 onward (Fig. 6A), although collagen type X protein was not detected by immunoblotting until day 21 (Fig. 6B). Interestingly, although the overall morphology was similar between Wnt-5a overexpressing and control cultures on day 21 (compare Fig. 3A,B; c–f), collagen type X expression was substantially reduced in the former; no collagen type X expression was detected in cells transfected with Wnt-7a (Fig. 6). Chfz-1 and Chfz-7 overexpression also resulted in a slight decrease in collagen type X expression (Fig. 6). Results from Northern blot analysis of collagen type X mRNA expression on day 21 were consistent with the protein results (Fig. 6C). These findings suggest that collagen type X expression could be down-regulated by the action of Wnt-related genes in the course of chondrocyte hypertrophy.

Alkaline Phosphatase Activity and Direct Red Staining for Matrix Mineralization

Alkaline phosphatase activity associated with matrix mineralization during chondrocyte

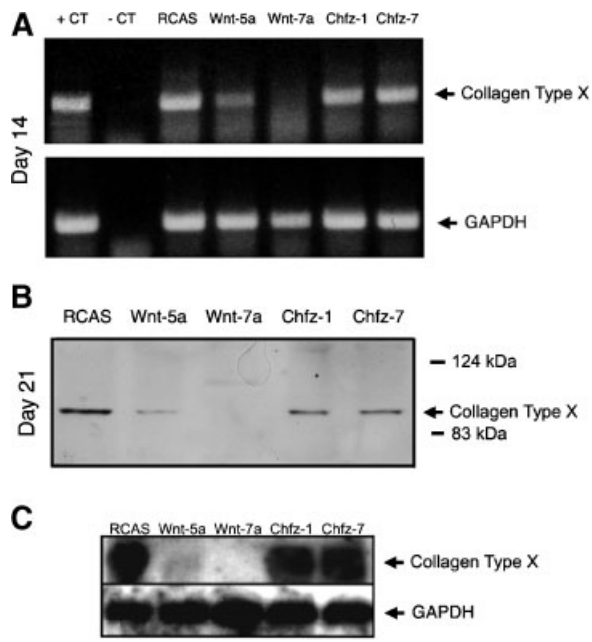

Fig. 6. Collagen type X expression in limb mesenchyme micromass culture. **A**: RT-PCR analysis on day 14. Cultures overexpressing Wnt-7a showed no collagen type X transcript, while cultures overexpressing Wnt-5a showed a decrease in transcript compared to RCAS control. Chfz-1 and Chfz-7 overexpressing cultures also showed a slight decrease in collagen type X expression compared to RCAS control. Positive control, day 21 untransfected limb mesenchyme micromass RNA; negative control, no RNA template. **B**: Western blot analysis on day 21. Collagen type X protein expression was detected in RCAS controls, and to a lesser extent, in cultures overexpressing Chfz-1 or Chfz-7. Overexpression of Wnt-5a resulted in a severe decrease in collagen type X expression, while cultures overexpressing Wnt-7a showed no collagen type X expression. **C**: Northern blot analysis on day 21. The profile of collagen type X mRNA levels was essentially similar to those in A and B.

maturation was next examined histochemically on histological sections. Enzyme activity in control cultures was clearly detectable by day 21 (Fig. 7A). Similar to the collagen type X results, all cultures overexpressing Wnt members exhibited less staining than RCAS empty control, despite the observation that all cultures except those overexpressing Wnt-7a appeared morphologically similar at this time point. This lack of correlation between alkaline phosphatase activity and phenotypic change is consistent with previous findings of Stott and Chuong [1997], who reported that retrovirally-mediated overexpression of alkaline phosphatase in chick limb micromass cultures resulted in a similar phenotype to control cultures, despite a 20-fold increase in alkaline phosphatase activity. Cultures overexpressing Wnt-5a (Fig. 7B) showed a pattern of positive staining similar to that of control cultures in that it was distributed relatively uniformly across areas of hypertrophy. However, cultures misexpressing Chfz-1 or Chfz-7 (Fig. 7D,E) appeared to have smaller, more localized areas of positive staining associated with discrete hypertrophic cells. Cultures overexpressing Wnt-7a consistently exhibited defined areas of positive staining localized to the middle of the tissue sections on day 21 (Fig. 7C), indicating that chondrocyte differentiation and maturation may not be a requirement for mineralized matrix deposition in this long-term chick limb mesenchyme cell culture system.

In contrast, staining with Direct Red for matrix mineralization showed that all cultures progressively mineralized as a function of culture time (from day 7 to day 21), with no apparent difference among RCAS control and cultures transfected with Wnt-5a, Chfz-1, or Chfz-7 (Fig. 7F). (Note, Wnt-7a overexpressing cultures consistently showed only non-specific background staining at all time points, and were thus omitted from Fig. 7F.)

Cell Proliferation and Apoptosis

Mature, hypertrophic chondrocytes typically exit the cell cycle and undergo apoptosis. All cultures exhibited a relatively high percentage of BrdU incorporation, indicative of cellular proliferative state, on day 7 (data not shown). As reported previously [Mello and Tuan, 1999], in control cultures positively stained cells decreased in number over time and became restricted to internodular regions. However, cultures overexpressing Wnt-5a or Wnt-7a both showed a higher percentage of positive cells compared to control and Chfz misexpressing cultures at day 21, and positive staining was localized to both internodular regions, as well as large cells located within the nodules in Wnt-5a overexpressing cultures (data not shown).

Apoptotic cells were detected by TUNEL staining beginning at day 7 and increased in number as a function of time in RCAS control cultures (Fig. 8A–C). Positive staining was localized to hypertrophic cells in all cultures, except for those overexpressing Wnt-5a (Fig. 8D–F) or Wnt-7a (Fig. 8G–I). In cultures overexpressing Wnt-7a, a relatively uniform distribution of TUNEL positive cells was seen throughout the culture across all time points (Fig. 8G–I); for Wnt-5a overexpressing cultures, substantially fewer apoptotic cells were seen in hypertrophic regions than all other cultures by day 21 (Fig. 8F). These results inversely paralleled the observation on cell proliferation, and provided further evidence for a delay or block of chondrocyte maturation caused by misexpression of Wnt-5a.

Expression Patterns of β-Catenin and N-Cadherin

In control cultures, Western blot analysis of total cellular β-catenin protein showed comparable levels throughout the 21-day culture period (Fig. 9A–C). Interestingly, although we previously observed unchanged cellular levels of β-catenin in Wnt-5a or Wnt-7a misexpressing cells during short-term (3 day) micromass cultures [Tufan and Tuan, 2001], in this study, overexpressing Wnt-7a or Wnt-5a significantly effected β-catenin level in long-term cultures. Thus, after day 7 (or day 14 and day 21), Wnt-5a overexpression resulted in a decrease in β-catenin levels compared to cells transfected with the RCAS-empty vector; on the other hand, Wnt-7a overexpression resulted in a dramatic increase in β-catenin levels compared to RCAS controls at all time points (Fig. 9A–C). Chfz-1 or Chfz-7 overexpression seemed to have no observable effect on total cellular β-catenin levels on day 7 (Fig. 9A). However, by day 14, cultures overexpressing Chfz-7 showed higher levels of β-catenin than control, while Chfz-1 appeared similar to control (Fig. 9B). Interestingly, at day 21, both Chfz-1 and Chfz-7 overexpression resulted in a decrease in β-catenin levels compared to RCAS control (Fig. 9C).

Previous studies have demonstrated a link between the stability of cadherin-dependent

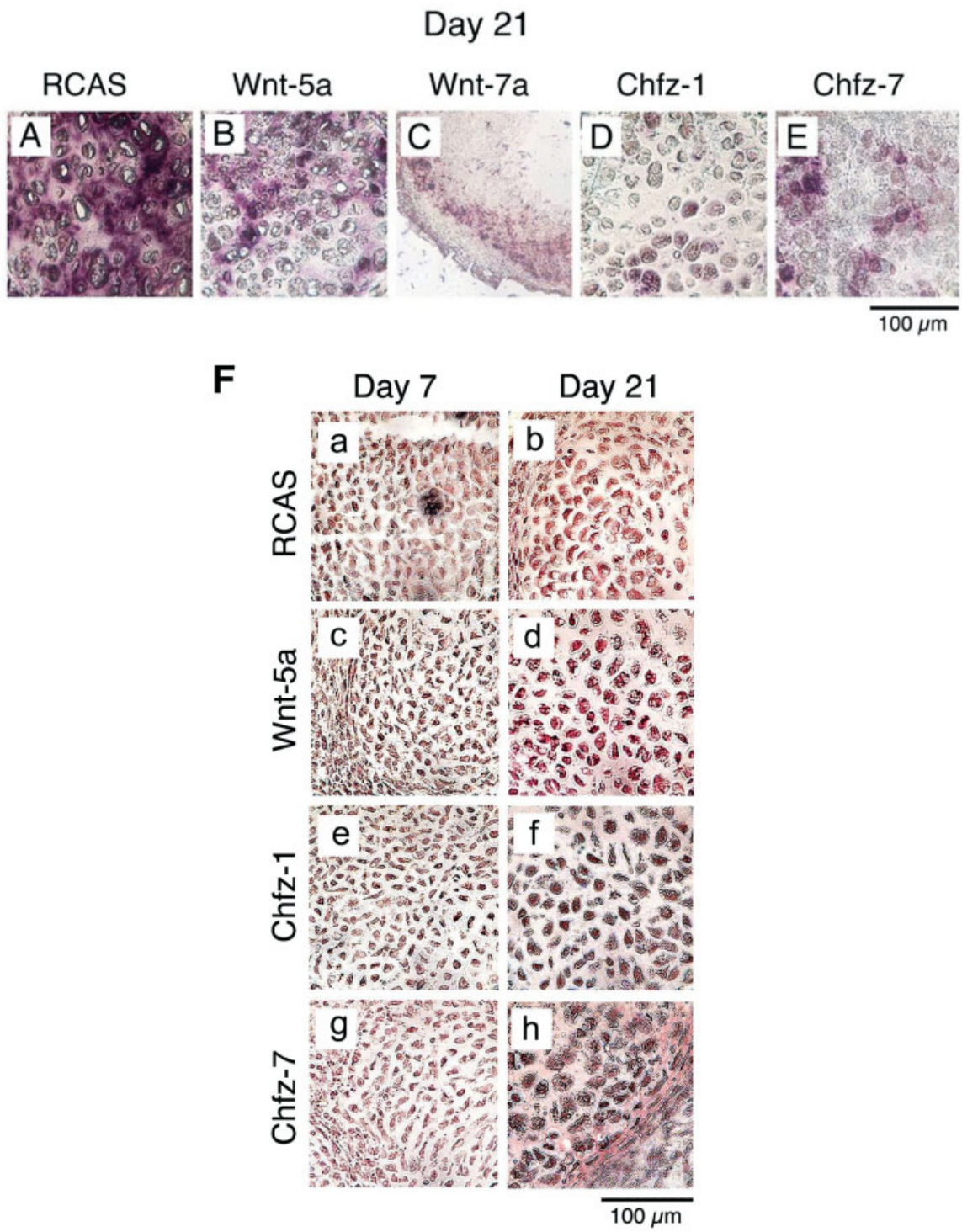

Fig. 7. Histochemical analysis of alkaline phosphatase activity and analysis of matrix mineralization by Direct Red staining in limb mesenchyme micromass cultures. **A**–**E**: Histochemical analysis of alkaline phosphatase activity. Cultures were stained on days 7, 14, and 21, and only day 21 specimens showed positive staining. RCAS controls (A) showed the most intense positive staining, followed by cultures overexpressing Wnt-5a (B). Cultures overexpressing Chfz-1 (D) or Chfz-7 (E) showed significantly reduced staining. Cultures overexpressing Wnt-7a (C) showed slight positive staining limited to a defined area near the center of the culture. **F**: Analysis of matrix mineralization by Direct Red staining. All cultures progressively mineralized as a function of culture time (from day 7 to day 21), with no apparent difference among RCAS control (**a** and **b**) and cultures transfected with Wnt-5a (**c** and **d**), Chfz-1 (**e** and **f**), or Chfz-7 (**g** and **h**).

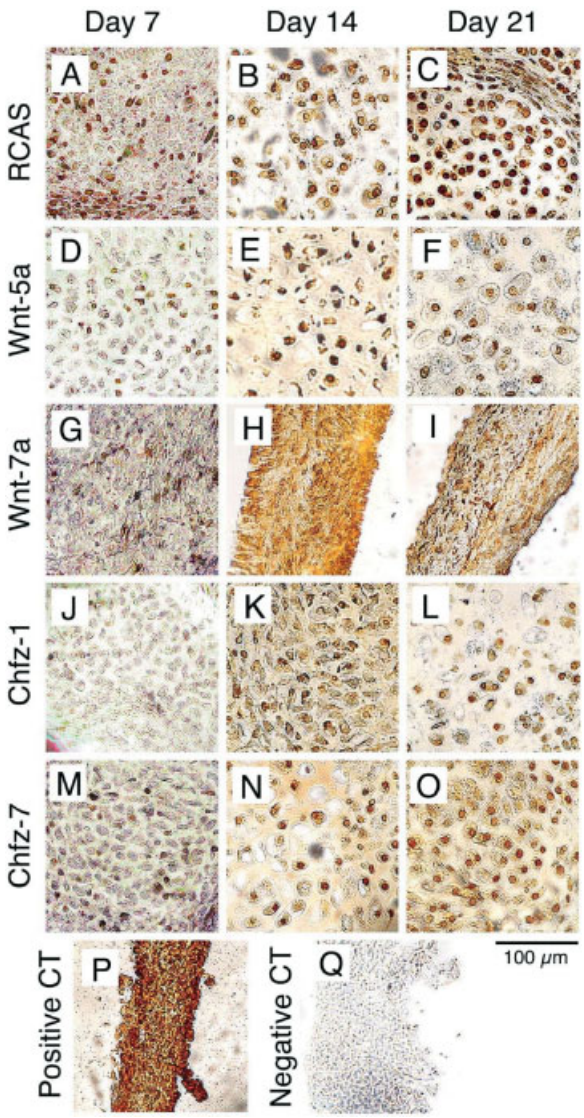

Fig. 8. Analysis of apoptosis in limb mesenchymal micromass cultures. Apoptotic cells were detected by transferase-mediated dUTP nick end labeling (TUNEL) staining. The presence of apoptotic cells was detected by day 7 in all cultures and, in general, the percentage of TUNEL-positive cells increased as a function of time. The apoptotic cells were localized to areas of hypertrophy in all cultures, except for those overexpressing Wnt-5a (**D**–**F**) or Wnt-7a (**G**–**I**). In cultures overexpressing Wnt-7a, a relatively uniform distribution of positive staining was seen across all time points (G–I). Wnt-5a misexpressing cultures showed the highest percentage of apoptotic cells at day 14 (E), and by day 21 (F), displayed fewer apoptotic cells in hypertrophic regions than the RCAS control (**C**) or other treated cultures (**I**, **L**, **O**). **P**: Positive control consisting of day 3 untransfected culture treated with DNase I to induce DNA strand breaks; (**Q**) negative control with primary antibodies omitted from the detection mix.

cell–cell junctions and the accumulation of their associated cytoplasmic proteins, the catenins [Dale, 1998; Giarre et al., 1998; Dierick and Bejsovec, 1999]. We next evaluated the effects of Wnt and Frizzled misexpression on total cellular N-cadherin protein levels. In cultures transfected with RCAS-empty vector, N-cadherin levels were low on day 7, but increased on days 14 and 21 (Fig. 9D–F), consistent with the findings of Tavella et al. [1994], who reported an increase in N-cadherin expression in areas of chondrocyte hypertrophy. At day 7, cultures overexpressing Wnt-5a or Wnt-7a showed an increase in N-cadherin expression, while cultures overexpressing Chfz-1 or Chfz-7 showed a decrease compared to control (Fig. 9D). By day 14, Wnt-7a overexpression had resulted in a dramatic increase in N-cadherin protein levels, while cultures overexpressing Wnt-5a showed a decrease in N-cadherin (Fig. 9E). Cultures overexpressing Chfz-1 or Chfz-7 also showed a slight decrease in N-cadherin levels compared to control cultures (Fig. 9E). By day 21, cultures transfected with Wnt-7a showed a slightly higher level of N-cadherin expression compared to control cultures, while cultures overexpressing Wnt-5a, Chfz-1, and Chfz-7 showed a slight decrease in N-cadherin levels (Fig. 9F).

DISCUSSION

The culturing of primary chick limb mesenchymal cells as high-density micromass recapitulates the process of endochondral ossification in vitro, including the condensation, differentiation, and maturation phases [Ahrens et al., 1977; Mello and Tuan, 1999]. Using a long-term (21 day) chick mesenchymal cell micromass system as described by Mello and Tuan [1999], we have begun to investigate the effects of misexpressing Wnt-5a, Wnt-7a, Chfz-1, or Chfz-7 on late chondrogenic events in vitro. The use of avian-specific retroviral constructs (RCAS), which are expressed for the entire duration of the culture period, allows us to determine the possible roles of Wnts and Frizzleds during these events, particularly in chondrocyte maturation and hypertrophy. Our results also confirm that cultures transfected with RCAS-empty vector control plasmids exhibited phenotypic and molecular similarities to the untransfected micromass cultures characterized by Mello and Tuan [1999], demonstrating the utility of this system to assess the effects of ectopic gene expression during cartilage maturation and hypertrophy.

Wnt-5a, Wnt-7a, Chfz-1, and Chfz-7 are expressed in distinct spatio-temporal patterns

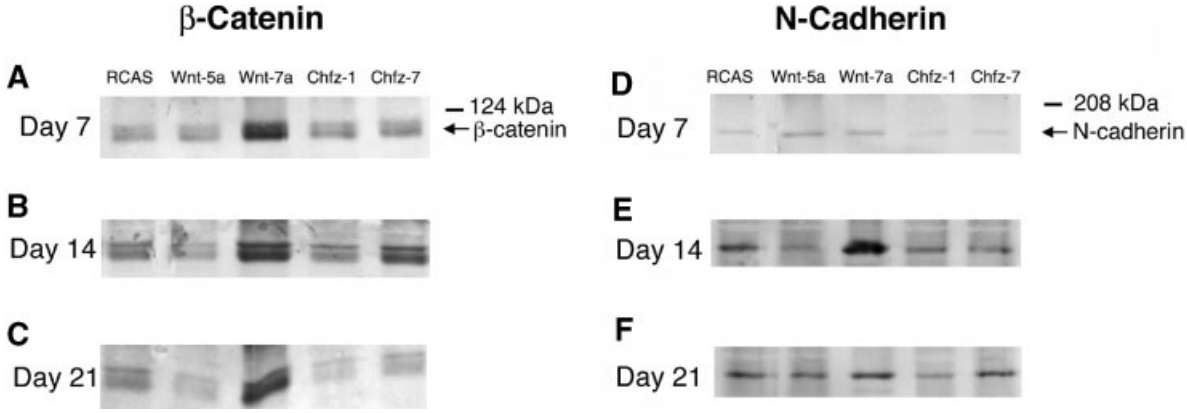

Fig. 9. β-Catenin and N-cadherin expression in limb mesenchyme micromass cultures. Whole cell protein extracts were analyzed by Western blotting. Equivalent protein load was analyzed on the basis of Ponceau S staining of filters prior to incubation with specific antibodies. **A**–**C**: β-Catenin. At day 7 (A), Wnt-7a overexpressing cultures showed a dramatic increase in β-catenin protein levels. At day 14 (B), cultures overexpressing Wnt-7a or Chfz-7 showed an increase in β-catenin expression compared to RCAS control, while cultures overexpressing Wnt-5a showed a decrease in β-catenin expression. Cultures overexpressing Chfz-1 showed comparable levels of β-catenin at this time point. At day 21 (C), cultures overexpressing Wnt-7a again showed an increase in β-catenin levels compared to RCAS control. Cultures overexpressing Wnt-5a, Chfz-1, or Chfz-7 showed low levels of β-catenin compared to control. **D**–**F**: N-Cadherin. At day 7 (D), Wnt-5a or Wnt-7a overexpressing cultures showed an increase in N-cadherin expression compared to RCAS control. At day 14 (E), cultures overexpressing Wnt-7a showed an increase in N-cadherin expression compared to RCAS control, while cultures overexpressing Wnt-5a showed a dramatic decrease in N-cadherin expression. Cultures overexpressing Chfz-1 or Chfz-7 showed slightly less N-cadherin expression than control at this time point. At day 21 (F), cultures overexpressing Wnt-7a showed slightly higher levels of N-cadherin than RCAS control, while cultures overexpressing Wnt-5a, Chfz-1, or Chfz-7 showed a slight decrease in N-cadherin.

within the developing chick limb [Dealy et al., 1993; Kengaku et al., 1997; Yang, 2003; Tufan et al., unpublished data], and have been implicated in the regulation of chick mesenchymal chondrogenesis [Kawakami et al., 1999; Hartmann and Tabin, 2000; Tufan and Tuan, 2001]. We have also recently detected, by whole-mount in situ hybridization, expression of these molecules even at late stages of limb cartilage development [Tufan et al., unpublished data]. (Note, the apparent lack of endogenous expression of Wnt-5a and Wnt-7a in Fig. 1 could be due to in sufficient cycle number, i.e., 25 cycles, used for PCR in this experiment.) Recent in vivo studies revealed that Wnt-5a misexpression delays the maturation of chondrocytes and decreases collagen type X expression even though the condensation and differentiation phases appeared to be unaffected [Kawakami et al., 1999; Hartmann and Tabin, 2000]. In this study, Wnt-5a overexpression caused a delay in the maturation of differentiated chondrocytes as shown by a decrease in Alcian Blue staining at day 7 (Fig. 3B; d). Although Alcian Blue staining appeared more similar to controls by day 14 (Fig. 3B; e), H/E staining revealed a poor hypertrophic phenotype for these cells at this time point (Fig. 3A; e). Similar to previous in vivo studies [Kawakami et al., 1999; Hartmann and Tabin, 2000], Wnt-5a overexpressing cultures showed decreased levels of collagen type X expression compared to controls (Fig. 6), as well as a decrease in alkaline phosphatase activity (Fig. 7). Wnt-5a has been identified as the primary member of the Wnt-5a class of Wnt molecules [Wong et al., 1994; Shimizu et al., 1997]. Members of this group do not exhibit the ability to transform mammalian epithelial cells in culture, and are not thought to signal primarily through the classical β-catenin/LEF-1 pathway. Instead, Wnt-5a signaling may be mediated through intracellular Ca^{2+} levels and PKC activation [Slusarski et al., 1997a,b; Miller et al., 1999; Scheldahl et al., 1999]. Hartmann and Tabin [2000] demonstrated that overexpression of β-catenin in vivo resulted in a phenotype opposite to that of Wnt-5a overexpression, i.e., accelerated cartilage maturation. Therefore, we would not expect to see any effect on β-catenin by Wnt-5a overexpression. However, although β-catenin levels appear similar to controls at day 7 (Fig. 9), there is a decrease in β-catenin compared to control as days 14 and 21. A recent study by Topol et al. [2003], which showed that Wnt-5a degrades β-catenin in both the inactive and active forms by an atypical Wnt signaling pathway, supports this finding. While further

investigation is needed to determine the phosphorylation state and localization (i.e., cytoplasmic or nuclear) of β-catenin, these data also suggest a possible antagonistic interaction of Wnt-5a with a Wnt member of the Wnt-1 class. Recent studies support this concept of antagonism between the different classes of Wnts [Kuhl et al., 2001; Ishitani et al., 2003]. Additional support for this comes from the results of Wnt-5a overexpression on N-cadherin levels (Fig. 9D–F). At day 7, cultures overexpressing Wnt-5a show a slightly elevated level of N-cadherin compared to RCAS control. This increase in N-cadherin expression, which is also visible in Wnt-7a overexpressing cells at this time point, may be a contributor to the slight delay in chondrocyte maturation evident at day 7 (Fig. 3B; d). N-Cadherin expression is upregulated in hypertrophic chondrocytes and osteoblast-like cells cultured in the presence of ascorbate and β-glycerophosphate, implicating a functional role for this protein in cartilage maturation [Tavella et al., 1994]. Furthermore, Wnt-5a misexpression caused a shift in the trends normally observed in the number and location of proliferating cells (data not shown) as well as those undergoing apoptosis (Fig. 8D–F). An alternative effect of Wnt-5a overexpression could be the stabilization of differentiated chondrocytes at a slightly mature stage of development, preventing further progression through endochondral ossification. Analysis of other genetic markers associated with apoptosis and mineralization (e.g., caspases, PTHrP, osteopontin, bone sialoprotein) would be helpful in determining whether Wnt-5a overexpression halts, or merely delays, cartilage development.

Wnt-7a is a member of the Wnt-1 class of Wnt molecules, and has been demonstrated to signal through the classical β-catenin/LEF-1 mediated pathway [Wong et al., 1994; Shimizu et al., 1997]. We [Tufan and Tuan, 2001; Tufan et al., unpublished data] and others [Rudnicki and Brown, 1997] have reported the chondro-inhibitory effects of Wnt-7a overexpression on chondrogenesis in vitro. In this study, cultures overexpressing Wnt-7a did not differentiate into chondrocytes, and retained the fibroblast phenotype throughout the duration of the culture period (Figs. 2D–F, 3A–B; g–i, and 5C). However, despite this observation, Wnt-7a did alter expression levels of β-catenin and N-cadherin (Fig. 9), and cultures overexpressing Wnt-7a exhibited some positive staining for alkaline phosphatase (Fig. 7). At all time points, cultures overexpressing Wnt-7a showed a dramatic increase in total cellular β-catenin protein levels compared to RCAS control, as well increased levels of N-cadherin (Fig. 9). Hartmann and Tabin [2000] have reported that constitutively expressed β-catenin results in an acceleration in chondrocyte maturation and hypertrophy. Similarly, a report by Tavella et al. [1994] has implicated a positive role for N-cadherin in the regulation of chondrocyte maturation. However, we see a severely inhibitory effect by Wnt-7a in our system despite an increase of β-catenin and N-cadherin protein levels. Interestingly, Hartmann and Tabin [2000] have reported that overexpression of Wnt-4, a member of the Wnt-1 class, results in the acceleration in cartilage maturation in vivo. In this study, we also observed that overexpression of Wnt-7a corresponds to an increase in Chfz-1 and Chfz-7 expression by day 21 as seen by RT-PCR (Fig. 1), two Frizzleds which are thought to act through the classical β-catenin/LEF-1 pathway [Scheldahl et al., 1999]. This biological response suggests the intriguing possibility that these two Frizzleds may be candidate receptors for Wnt-7a, a hypothesis that remains be proven. Taken together, these data suggest the intriguing possibility of a chondro-enhancing role for Wnt-7a during late events of cartilage development provided that the cells have already passed the differentiation stage. It would be of interest to induce the overexpression of Wnt-7a at various time points in differentiated chondrocytes (i.e., post-day 7) during long-term micromass cultures. It is possible that the complete inhibition of chondrogenesis by constitutive, ectopic expression of Wnt-7a at the pre-condensation stage prevents any later roles for endogenous Wnt-7a signaling during cartilage development from being detected in our system.

We next examined the effects of Chfz-1 and Chfz-7 misexpression. There has been a question of whether Frizzled misexpression results in an amplification of Wnt signal, or instead competes for intracellular signaling components and thus results in a loss of function effect. A previous study using these same RCAS–Chfz constructs [Hartmann and Tabin, 2000] reveals the latter as the more likely possibility, as the misexpression of the wild type Frizzled constructs mimicked the misexpression of mutant Chfz-1 and Chfz-7 constructs

containing C-terminal truncations. This phenomenon has also been observed in the *Drosophila* eye, where ectopic expression of Frizzled resulted in effects identical to those of known loss of function studies [Tomlinson et al., 1997; Zhang and Carthew, 1998; Tomlinson and Struhl, 1999]. We have observed the same effects when we overexpressed Chfz wild type plasmids or the dominant negative mutants in short-term micromass cultures in another study [Tufan et al., unpublished data]. Furthermore, misexpression of Chfz-1 or Chfz-7 resulted in a decrease of both β-catenin and N-cadherin expression by day 21 compared to control, consistent with the idea that overexpression of the receptors binds intracellular signaling components and thus antagonizes the endogenous Wnt β-catenin/LEF-1 pathway. Therefore, we conclude that misexpression of Chfz-1 and Chfz-7 in our system mimics a dominant negative effect, and this is actually a loss-of-function study. We previously observed an inhibitory effect of Chfz-7 misexpression on chondrocyte differentiation, while misexpression of Chfz-1 seemed to have no effect on early chondrogenic events [Tufan et al., unpublished data]. Here, misexpression of Chfz-7 resulted in an initial delay in chondrocyte maturation as assessed by Alcian Blue staining. However, these cultures appear phenotypically similar to controls by day 21. Molecular analysis of these cultures reveals a decrease in collagen type X expression as well as alkaline phosphatase activity, suggesting that the recovery is incomplete. Cultures misexpressing Chfz-1 appeared phenotypically similar to control cultures at each time point (Figs. 3 and 4). However, Chfz-1 misexpressing cultures also exhibited a decrease in collagen type X expression (Fig. 6) and a more significant decrease in alkaline phosphatase activity than misexpression of Chfz-7 (Fig. 7). Frizzled misexpression also altered β-catenin and N-cadherin expression levels (Fig. 9). Cultures overexpressing Chfz-1 or Chfz-7 exhibited a decrease in total β-catenin as well as N-cadherin levels on day 21, consistent with the suggestion that β-catenin and N-cadherin are positive regulators of chondrocyte maturation. Taken together, these results suggest a positive regulatory role for these Frizzled receptors in vivo, and suggest that Wnt signaling involved in late events is transduced down the classical pathway. Further investigation is needed to demonstrate specific Wnt–Frizzled interactions during chondrogenesis, and to determine if Wnt signaling is mediated at the level of the ligand or the receptor during cartilage development.

In conclusion, our current observations provide strong implication of the involvement of Wnt signaling in the maturation and hypertrophy programs of cartilage development. This study also represents the first to use a long-term in vitro micromass culture system to investigate the effects of ectopic gene expression on late events of limb cartilage development. This system should prove invaluable for future studies examining the effects of other exogenous factors on multiple aspects of the program of endochondral ossification.

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