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Transcription Factor ERG and Joint and Articular Cartilage Formation during Mouse Limb and Spine Skeletogenesis

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Abstract

Articular cartilage and synovial joints are critical for skeletal function, but the mechanisms regulating their development are largely unknown. In previous studies we found that the *ets* transcription factor ERG and its alternatively-spliced variant C-1-1 have roles in joint formation in chick. Here, we extended our studies to mouse. We found that ERG is also expressed in developing mouse limb joints. To test regulation of ERG expression, beads coated with the joint master regulator protein GDF-5 were implanted close to incipient joints in mouse limb explants; this led to rapid and strong ectopic ERG expression. We cloned and characterized several mammalian ERG variants and expressed a human C-1-1 counterpart (hERG3Δ81) throughout the cartilaginous skeleton of transgenic mice, using Col2a1 gene promoter/enhancer sequences. The skeletal phenotype was severe and neonatal lethal, and the transgenic mice were smaller than wild type littermates and their skeletons were largely cartilaginous. Limb long bone anlagen were entirely composed of chondrocytes actively expressing collagen IX and aggrecan as well as articular markers such as tenascin-C. Typical growth plates were absent and there was very low expression of maturation and hypertrophy markers, including Indian hedgehog, collagen X and MMP-13. The results suggest that ERG is part of molecular mechanisms leading chondrocytes into a permanent developmental path and become joint forming cells, and may do so by acting downstream of GDF-5.

Keywords

ERG transcription factor; articular chondrocytes; limb and spine skeletogenesis; GDF-5; extracellular matrix

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Introduction

During limb skeletogenesis, chondrocytes follow two developmental paths. The relatively few chondrocytes forming at each epiphyseal end of long bone anlagen develop into permanent articular chondrocytes. These cells exhibit a stable phenotype, produce and deposit an abundant and resilient extracellular matrix composed of aggrecan, collagen II and other macromolecules, and maintain tissue and synovial joint structure and function though life. Instead, the more numerous chondrocytes constituting the metaphyseal and diaphyseal portions of the long bone templates are transient cells, are organized in growth plates in which they undergo proliferation, maturation, hypertrophy and major changes in gene expression, and are eventually replaced by bone and marrow cells via endochondral ossification. Growth plate chondrocytes exert their roles up until the end of puberty at which point they disappear upon completion of skeletal growth. The critical importance of articular and growth plate chondrocytes for skeletal organization, growth and function is widely recognized and acknowledged, and both cell types continue to attract strong research activity at the basic and biomedical level (Archer et al., 1999; Kronenberg, 2003; Lefebvre and Smits, 2005). On the other hand, the mechanisms by which chondrocytes handle their developmental choices at early embryonic stages and give rise to permanent articular or transient growth plate cartilage remain largely unclear, particularly at the molecular level (Archer et al., 2003; Pacifici et al., 2005).

ERG belongs to the *ets* gene family of transcription factors (Sharrocks, 2001). The current 26 family members are involved in a variety of cellular and developmental processes and when mutated or mis-expressed, can cause pathologies (Muthusamy et al., 1995; Sharrocks, 2001; Tondravi et al., 1997). *Ets* proteins share a highly conserved 85-amino acid domain (the ETS domain) that binds to the consensus DNA core sequence 5′-GGA(A/T)-3′ (Karim et al., 1990). Individual *ets* proteins can select specific nucleotides over an 11 bp sequence centered around the core sequence. Additional specificity is provided by interactions with other transcription factors (Verger et al., 2001; Verger and Duterque-Coquillaud, 2002), phosphorylation and conformational changes consequent to interactions with other factors (Papoutsopolou and Janknecht, 2000; Sharrocks, 2001; Yang et al., 2001). The ETS domain is a variant of the winged helix-turn-helix motif and displays three α-helices and four β-sheets, with the main protein-DNA contacts provided by residues located along the third α-helix. Though the ETS domain is highly conserved with the family, *ets* proteins differ from each other in other domains (such as absence or presence of the Pointed/SAM domain) and are thus distinguished in sub-families (Sharrocks, 2001; Verger and Duterque-Coquillaud, 2002).

ERG belongs to the Erg/Fli-1 sub-family. ERG has been widely studied for its involvement in human cancers following gene translocation to form fusion proteins (Shimizu et al., 1993). ERG is also linked to normal processes such as mesoderm formation (Vlaeminck-Guillem et al., 2000) and is found to form functional complexes with Jun/Fos, with the resulting ternary complexes regulating expression of genes such as metalloprotease-1 (MMP-1) and MMP-3 (Buttice' et al., 1996). Most relevant to the present study are the original studies of Dhordain et al. providing the initial evidence that ERG is expressed at sites of future synovial joint formation in chick embryo limbs (Dhordain et al., 1995). These data were confirmed and extended by Hurle and co-workers showing that joint malformations provoked by ectopic expression of transforming growth factor β-family members were associated with defective ERG expression (Ganan et al., 1996). Following the lead of these groups, we carried out additional studies in chick embryos (Iwamoto et al., 2001; Iwamoto et al., 2000). We found that ERG was not only expressed at the onset of joint formation, but persisted once the articular layer had developed further. Aware of the fact that *ets* proteins can be alternatively-spliced (Duterque-Coquillaud et al., 1993), we carried out additional analyses and cloned a variant missing an 81-bp segment (exon 5) in the central portion of ERG. We named this variant C-1-1 and found that it was preferentially, albeit not exclusively, expressed in most epiphyseal pre-

articular/articular chondrocytes in developing long bones. When we mis-expressed C-1-1 in developing chick limbs, we observed that C-1-1 was able to impose a stable, immature and articular-like phenotype onto the entire limb chondrocyte population, effectively blocking maturation and endochondral ossification (Iwamoto et al., 2001; Iwamoto et al., 2000). These and other data led us to conclude that ERG is part of molecular mechanisms for joint and articular cartilage formation in chick embryos.

The present study was conducted to extend our analyses to mammalian systems, specifically to identify mammalian counterpart(s) of avian C-1-1 and determine its biological properties and roles during limb skeletogenesis. In addition, we searched for upstream regulators that could induce and restrict ERG gene expression to developing joint cells. We focused on the bone morphogenetic protein family member GDF-5 that is thought to represent a master regulator of joint formation (Storm and Kingsley, 1999). GDF-5 expression is particularly prominent in the interzone, a three-layered mesenchymal structure that demarcates and constitutes the joint forming area and whose cells are thought to give rise to joint tissues in developing long bones (Holder, 1977; Ito and Kida, 2000). We describe here the cloning of a human ERG variant homologous of avian C-1-1 and its functional characterization by transgenic expression in the cartilages of mouse embryos using Col2a1 promoter/enhancer sequences. We report also that there is close spatio-temporal expression of both ERG and GDF-5 in developing mouse embryo joints and that GDF-5 is a rapid and effective inducer of ERG expression.

Materials and methods

Cloning of mammalian ERG variants

cDNA clones encoding mouse ERG variants were isolated from a E15.5 whole mouse embryo plasmid library (GibcoBRL) using Gene Trapper cDNA Positive Selection System according to manufacturer's instructions. Briefly, 5 μg of double strand cDNA pool prepared from the cDNA library was digested with Gene II polymerase and Exo III nuclease to generate single strand DNA pool (ssDNA pool). The ssDNA pool was hybridized with a biotinylated antisense mouse ERG oligo (5′-GCTGGAGTGGGCGGTGAAAGAATA -3′) common to all variants. Hybridized DNAs captured by magnetic streptavidin beads were converted to double strand DNA with gene-specific primer (5′-GCCGACATTCTTCTCTCACATCTC-3′) and then used to transform DH10B (GibcoBRL). After transformation, we carried out colony PCR with the primer pair 5′-GGAGAGGAAGAGCAAGCCCAACAT-3′ and 5′-TCGGGGAAGCAAAGAAACTGGAA-3′ which amplify *ets* DNA binding domain; this resulted in the identification of 19 *ets*-domain containing clones. Clones were sequenced and their predicted structures are shown in Fig.1A.

To obtain human ERG cDNA variants, we used a combination of 5′ and 3′RACE techniques. We first constructed a library of adaptor-ligated double-strand cDNAs with total RNA from fetal brain and cartilage (kindly provided by Dr. J. Miki, Tsukuba, Japan) using the Marathon cDNA amplification kit according to the manufacturer's protocol (Clontech, Palo Alto, CA). The library was used as a template for 3′- and 5′-RACE reactions. We first performed 3′-RACE reaction with adaptor primer (AP1) included in the kit and with a gene specific primer (5′-GAGAGCGGAAGAGCAAACCCAACA-3′) designed within *ets*-DNA binding domain of human ERG2 (M17254) (Rao et al., 1987). We then performed 5′-RACE reaction with AP1 primer and a gene specific primer (5′-TCATCTTGCACAGTTCCTTCCCATC -3′) designed within the SAM domain. PCR amplified 3′ and 5′ RACE products were subcloned into pGEM-T vector (Promega) and sequenced. Entire coding sequence of hERG3 and hERG3Δ81 were then amplified by PCR with 5′-GACATGATTCAGACTGTCCCGG -3′ and 5′-CCGCCAGGTCTTTAGTAGTAAGTGC -3′ and subcloned to pGEM-T vector.

Construction of recombinant retroviral vectors

Entire coding sequences of mERG, mERGΔ72 and mERGΔ69 were amplified by PCR from respective cDNA templates DNA using gene specific primers 5′-GCATTATGGCCAGCACTATTAAGGAGG -3′ and 5′-CCTAGTAGTAGGTGCCCAGGTGAGAGG -3′, subcloned to pGEM-T vector and sequenced. Full length mouse and human ERG variants in pGEM-T vector were released by SpeI and Not I digestion and subcloned into Spe I/Not I sites of RCASBP(B)L-44 vector (Iwamoto et al., 2003). Dermal chicken embryo fibroblasts from virus-free white leghorn 11 day-old embryos (SPAFAS) were cultured in medium 199 containing 10% fetal bovine serum and transfected with retroviral plasmid vectors by use of FuGENE6 transfection reagent (Roche Diagnostic Inc). Recombinant viral particles present in the medium were concentrated by ultracentrifugation (25,000 rpm for 3 h) and used to infect freshly isolated chondrocytes. By 1 and 3 weeks of culture, over 90% of the chondrocytes were infected as revealed by immunostaining for viral coat protein (Iwamoto et al., 2003). Insert-less control viral particles were produced and isolated in the same manner. Immature chondrocytes from the caudal portion of Day 17 chick embryo sterna were cultured in high-glucose DMEM containing 10% FBS. Culture medium was changed every other day after day 5 and cells were passaged weekly. At 1 and 3 weeks in culture, total RNA was collected by Trizol and used for RT-PCR analysis using primer pairs described previously (Iwamoto et al., 2003). APase activity associated with the cell layer was measured using p-nitrophenyl phosphate (pNP) as a substrate (Iwamoto et al., 2003).

Generation of transgenic mice

DNA fragments encoding an entire coding sequence of hERG3Δ81 were excised from pGEM-T vector by Spe I/Not I digestion and cloned into the Not1 site of a Col2a1-based expression vector by blunt end (Ueta et al., 2001). Synthetic Nar I site was introduced into a Hind III site of the above vector. The resulting vector contains hERG3Δ81 expression unit including 5′-Nar I site-Col2a1 promoter (nucleotide 1940-2971 of M65161), β-globin intron cassette, CA-LEF, SV40 poly A, Col2a1 enhancer (4930-5571 of M65161), Nar I site-3′ in pNASSβ backbone (Clontech, California, USA). The expression unit of hERG3Δ81 was excised by Nar I digestion, purified and injected into pronuclei of fertilized eggs from F1 hybrid mice (C57BL/6 X C3H). Transgenic embryos were identified by PCR and immunohistochemistry. Genomic DNA was prepared from liver tissue and subjected to PCR using primers 5′-TGGTCATCATCCTGCCTTTCTC-3′ and 5′-GGAAGTCATCCTTTGTCATCTTGC-3′ TGC AGC TTT ATC CAG GCT GGT CAG-3′ and 5′-CAC CCA TCT CAT GCT CCA TCA TCA TAG G-3′ that amplify 316 bp fragment of part of β-globin intron and hERG3Δ81. Transgene expression at protein level was analyzed by immunostaining with anti-human ERG antibody (ERG-1//2/3 (C-20), sc-353)(Santa Cruz Biotechnology, CA) (Iwamoto et al., 2000). Sixty six out of 577 embryos were transgene positive by PCR genotyping, and transgene over-expression was observed in 11 embryos that were examined in the present study.

Anatomical, histological and immunohistochemical analyses

Whole skeletons of E18.5 embryos were stained with alizarin red S and alcian blue (Ueta et al., 2001). MicroCT analysis was carried out by a centralized facility, using a Scanco μCT40 apparatus. For histology, E18.5 tissues fixed in 4% paraformaldehyde/0.1M phosphate buffer were embedded in paraffin and sections stained with hematoxylin and eosin. For immunohistochemistry, sections were de-masked by treatment with 0.2% pepsin in 0.02N HCl for 15 min at 37°C, and were incubated with rabbit anti-mouse tenascin-C antibody (Ueta et al., 2001) or rabbit anti-human ERG (Santa Cruz Biotechnology) for 16 h at 4°C. The ERG antibodies react with every ERG variant. After rinsing, sections were incubated with biotinylated anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) and Cy3-conjugated

Streptavidin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA); each incubation was for 30 min at room temperature.

Explant cultures

Autopods were isolated from E12.5-E13.5 mouse embryos and day 5.5 chick embryos and placed in standard serum free organ cultures. At the moment of isolation, specimens displayed cartilaginous primordia of digits. A single heparin Sepharose bead presoaked for 1 hr with recombinant GDF-5 (10 μg/ml) was microsurgically implanted in proximity of the cartilaginous elements; companion specimens received a bead precoated with BSA as control (Koyama et al., 1999). All samples were incubated for 6 to 24 hrs and were then processed for whole mount detection of ERG expression (Shimo et al., 2005), using full length chick or mouse ERG probes that react with every variant as described (Iwamoto et al., 2000).

In situ hybridization

Tissue section in situ hybridization was carried out using digoxigenin-conjugated or ^{35}S- or ^{33}P-labeled riboprobes (Iwamoto et al., 2000; Ueta et al., 2001). pGEM-T vector encoding full length mERG was used to generate riboprobes common to all mouse ERG variants. PGEM-T vectors encoding GDF-5 (1321-1871 of NM_008109) and PRG4/Lubricin (41-2646 of AB034730) were used to generate respective probes. MMP-13 and other probes were as described (Jimenez et al., 1999; Tamamura et al., 2005).

Results

Cloning and characterization of mammalian ERG variants

Chick, mouse and human ERG genes have similar structure and organization. Each gene is transcribed from two transcriptional start sites and the alternative use of 3 exons in the variable (V) region (81, 69 and 72 bp long, respectively) generates multiple variants (Fig. 1) (Duterque-Coquillaud et al., 1993;Rao et al., 1987). As detailed in our previous studies (Iwamoto et al., 2001;Iwamoto et al., 2000), two main ERG variants are expressed in developing chick limbs: chERG that contains all 3 exons of the V region; and C-1-1 that lacks the 81 bp segment (Fig. 1A). To identify ERG variants expressed in mouse, we screened mouse embryo cDNA libraries and cloned several ERG variants including: (i) mERG corresponding to chERG; (ii) mERGΔ72 that lacks the 72 bp segment; and (iii) mERGΔ69 that lacks the 69 bp segment (Fig. 1A). Three other variants containing a longer 5′ un-translated region (AB073078, AB073079 and AB073080) had previously been deposited in the NCBI database. Thus, our screens did not lead to the identification of a mouse homologous of chick C-1-1 lacking the 81 bp only.

In concurrent studies, we made use of 5′ and 3′ RACE procedures with cDNA templates prepared with RNA from human cartilage and brain to characterize human ERG variants. Clones were sequenced and used to design new primers for amplification and cloning of the entire coding sequences. These approaches led us to identify several human ERG variants including: (i) hERG corresponding to chERG and mERG; (ii) hERG3 containing a longer 5′ un-translated region and corresponding to mERG3; and (iii) hERG3Δ81 that lacks the 81 bp segment and is thus similar to C-1-1 in its coding region, but contains a longer 5′ un-translated region (Fig. 1A).

As an initial characterization of functional properties of mouse and human ERG variants, we constructed avian retroviral RCAS expression vectors encoding mERG, mERGΔ72, mERGΔ69, hERG3 or hERG3Δ81 and tested each construct in primary cultures of chick sternum chondrocytes, a widely used system that permits rapid and effective evaluation of transgene effects on chondrocyte phenotype and maturation (Gibson and Flint, 1985; Iwamoto et al., 1993). Aliquots of freshly isolated chondrocytes were infected with each of the RCAS

viruses and plated in standard monolayer cultures; companion control cells received equal amounts of insert-less RCAS virus. After 1 week of culture, the control cells displayed a characteristic and relatively small cell size, strong gene expression of tenascin-C (TN-C) that is a marker of permanent and articular chondrocytes (Pacifici et al., 1993; Savarese et al., 1996), and very low and barely detectable expression of collagen X which is a marker of maturing hypertrophic chondrocytes (Fig. 1B, lane 1). By 3 weeks, TN-C expression had become undetectable and collagen X had increased significantly (Fig. 1B, lane 2) and the cells had increased in size (not shown), traits indicating that the cells were undergoing maturation and hypertrophy. When we examined 3 week-old cultures over-expressing RCAS-encoded mammalian ERG variants, none expressed collagen X at appreciable levels and each of them strongly expressed TN-C (Fig. 1B, lanes 3-7). We also measured alkaline phosphatase (APase) activity that is another marker of chondrocyte maturation and hypertrophy. Whereas APase activity was strong and easily detectable in 3 week-old control cultures, APase activity was consistently low in each mammalian ERG over-expressing culture (Fig. 1C). Together, the data indicate that mouse or human ERG variants have similar activities, stabilize chondrocyte function, inhibit chondrocyte progression toward maturation and thus favor a permanent articular-like phenotype.

ERG expression in developing mouse embryo limbs

The above conclusion implies that mammalian ERG should be expressed in developing permanent chondrocytes in vivo. To test this prediction, we carried out in situ hybridization analyses using serial longitudinal sections of E13.5 to E18.5 mouse embryo hind limbs. Sections were hybridized with riboprobes encoding: (a) full-length mERG recognizing every variant; (b) collagen IX that is a major cartilage matrix component and a typical phenotypic trait of functional chondrocytes; and (c) GDF-5 and lubricin/PRG4 that are markers of developing joint and articular chondrocytes (Rhee et al., 2005; Schumacher et al., 1994; Storm and Kingsley, 1999). In E13.5 embryos, the incipient knee joint was fairly well organized and the flanking tibia and femur elements were fully cartilaginous, and the more distal tarsal and phalageal joints and skeletal elements were developmentally younger (Fig. 2 A). ERG expression was prominent in developing knee joint (Fig. 2B, arrow) and incipient tarsal-phalangeal joints (Fig. 2B, arrowheads). GDF-5 was expressed in essentially super-imposable patterns (Fig. 2C, arrow and arrowheads, respectively). By E15.5, ERG and GDF-5 continued to exhibit comparable, preferential and quite strong expression in every limb joint, including distal tibial, ankle and phalangeal joints (Figs. 2 D-E). By E18.5, gene expression of ERG and GDF-5 was somewhat lower, but had become more clearly localized to the 5 or 6 layers of articulating chondrocytes abutting the synovial cavities (Figs. 2 G-I, arrowheads), a finding quite evident when viewed at higher magnification (Figs. 2 K-L). The same ERG-positive articulating chondrocytes strongly expressed lubricin (Figs. 2 M-N), attesting to the fact that they were acquiring and displaying characteristics of functional articular chondrocytes. ERG expression patterns were quite distinct from those of collagen IX that instead characterized the entire cartilaginous portions of limb elements (Fig. 2J).

To determine whether ERG is expressed in other joints, we examined the developing spine. Indeed, ERG as well as GDF-5 were strongly co-expressed in nascent inter-vertebral joints and more specifically in developing annulus fibrous (a permanent fibro-cartilage structure) and endplates (Fig. 3 A-F, arrows).

ERG induction by GDF-5

Given that ERG expression is closely associated with GDF-5 expression, it is possible that GDF-5 may actually induce/regulate ERG expression. To test this hypothesis, we isolated autopods (paws/feet) from E12.5-13.5 mouse embryos and Day 5.5 chick embryos and placed them in organ culture. These explants grow well in medium-defined conditions and continue

their development over time. At the moment of isolation, both murine and avian specimens contain cartilaginous templates of digits that are still largely un-interrupted at each future joint site; joint formation continues over time in culture. A single Affi-blue bead pre-soaked in recombinant GDF-5 (10 μg/ml) was microsurgically implanted in proximity of incipient metatarsal-phalangeal joints; companion control specimens received a bead pre-coated with vehicle (BSA). Cultures were re-incubated and examined over time from bead implantation by whole mount in situ hybridization. Indeed, within 6 hr of treatment, strong ERG expression visible as a purple hybridization signal was induced around the GDF-5 soaked bead (Figs. 4 B,D, arrows); control beads had no effect (Figs. 4 A,C). Strong ERG expression persisted at the other time points examined (not shown).

Transgenic analysis of mammalian ERG properties

To further characterize the biological properties of mammalian ERG, we created transgenic mice expressing hERG3Δ81 in developing cartilage under the control of mouse Col2a1 promoter/enhancer sequences. We focused on this human variant since it is the one most closely resembling avian C-1-1; the Col2a1 sequences are the ones we previously used to express isoforms of transcription factor Runx2 in the developing cartilaginous skeleton of transgenic mice (Ueta et al., 2001). Constructs were microinjected into the pronuclei of fertilized eggs by a centralized facility, and a total of 11 transgenic F0 mouse embryos were obtained and identified by presence of transgene DNA in cartilage (five embryos were examined by histology, histochemistry and in situ hybridization; 4 embryos were examined by alcian blue/alizarin red staining; and 1 embryo was subjected to soft x-ray and μCT analysis). To verify transgene expression, sections of E18.5 wild type and transgenic limb cartilages were processed for immunostaining with an ERG antiserum that recognizes every variant. Endogenous ERG protein in wild type tibias was visible in most epiphyseal articulating chondrocytes (Fig. 5A, arrows) and peri-epiphyseal perichondrium (Fig. 5A, arrowheads) as expected, whereas ERG was widespread in transgenic tibial chondrocytes (Fig. 5B). Higher magnification view showed that ERG staining was largely restricted to the nucleus (Fig. 5A, inset) and that the majority of the transgenic chondrocytes were positive (Fig. 5B inset). Not all chondrocytes were positive since the F0 embryos used here were most likely mosaic.

Mice over-expressing hERG3Δ81 died at birth from respiratory failure. As exemplified by analysis of E18.5 embryos stained with alizarin red and alcian blue, the transgenic embryos were much smaller and their trunk and limb skeletal elements were hypo-mineralized and stained poorly with alizarin red (Fig. 6D) compared to wild type littermates (Fig. 6A). Soft x-ray analysis confirmed the reduced levels of mineralization in transgenic vertebral column, ribs and limbs (Fig. 6 E-F) relative to wild type (Fig. 6 B-C). In addition, the transgenic cranium was smaller than wild type.

Serial sections of wild type and transgenic forelimbs were processed for histology to analyze the phenotype, maturation process and growth plate organization. Wild type E18.5 long bones such as the humerus displayed a typical structure consisting of a prominent epiphyseal cartilaginous cap (where a secondary ossification center will eventually form) and a well-organized growth plate in the metaphysis (Fig. 7 A,C). Instead, the transgenic humerus was disorganized (Fig. 7 B,D). The epiphyseal and metaphyseal portions consisted of a uniform cartilaginous tissue in which the chondrocytes, regardless of location, had a small and fairly uniform diameter and were not organized in a typical growth plate (Fig. 7 B,D). The diaphyseal portion contained bone-like tissue, but the tissue appeared fibrotic and very poor in marrow (Fig. 7D). To extend these observations, sections were stained with the proteoglycan matrix dye Safranin-O. This histochemical procedure showed that in wild type humerus the transition from hypertrophic cartilage to endochondral bone was gradual, and long stands of positively-staining hypertrophic cartilage intermingled with marrow and bone trabeculae forming a

primary spongiosa (Fig. 7 E,G, arrowheads) as expected (Poole et al., 1982). In contrast, the transition from cartilage to fibrous/bony tissue in transgenic humerus was abrupt and there was no obvious intermingling of cartilage with underlying tissues and virtual lack of primary spongiosa (Fig. 7 F,H). Analysis by μCT of companion specimens fully confirmed these observations (Fig. 7 I-J). Clear strands of mineralized tissue occupied the metaphyseal-diaphyseal region of wild humerus (Fig. 7I, arrowheads), but no such strands were visible in transgenic humerus (Fig. 7J). Interestingly, the cortical bone was essentially unaffected and quite similar to wild type (Fig. 7 I-J).

To characterize the chondrocyte phenotype, serial sections were processed for in situ hybridization. Gene expression of typical chondrocyte markers such as aggrecan and collagen IX was equally strong in wild type and transgenic humeri, indicating that the transgenic cells were functional and were not experiencing unexpected negative effects due to transgene expression (Fig. 8 A-B and F-G). However, expression of markers of chondrocyte maturation and hypertrophy, including Indian hedgehog (Ihh), collagen X and MMP-13, were all markedly reduced in transgenic (Fig. 8 H-J) versus wild type (Fig. 8 C-E). In good correlation, the transgenic chondrocytes expressed the articular chondrocyte marker tenascin-C broadly and strongly (Fig. 9B), whereas this protein was expressed in epiphyseal chondrocytes, bone and periosteum in wild type humerus as expected (Fig. 9A).

Discussion

In this study, we demonstrate that ERG expression characterizes developing synovial joints in mouse embryo limbs as we previously found in chick. The factor is initially expressed in the murine mesenchymal interzone, and expression continues in epiphyseal pre-articular/articular chondrocytes over increasing developmental time. ERG is co-expressed with the master joint regulator GDF-5 in limbs as well as in intervertebral joints, and we show that GDF-5 is actually a rapid inducer of ERG expression. Transgenic analysis reveals that human ERG maintains chondrocytes in a differentiated state characterized by strong expression of aggrecan, collagen IX and tenascin-C, but inhibits their progression toward hypertrophy and their organization into growth plates. Together, the data provide further support for the conclusion (Iwamoto et al., 2001; Iwamoto et al., 2000) that ERG is an important component of mechanisms of synovial joint formation and would in particular act to regulate the developmental behavior of most-epiphyseal chondrocytes and help them to acquire a permanent articular chondrocyte phenotype.

ERG and its variants

Given that ERG and other *ets* family members are known to be expressed in alternatively-spliced forms (Duterque-Coquillaud et al., 1993; Sharrocks, 2001), it is not surprising that we cloned several ERG variants from mouse and human tissues. The hERG3Δ81 variant lacks a 81 bp domain (corresponding to exon 5) in the V protein region and thus resembles avian C-1-1, though it contains a longer 5′ un-translated region likely due to usage of the upstream transcription start site. A homologue of C-1-1/ hERG3Δ81 was previously cloned and characterized in Xenopous (Baltizinger et al., 1999) and thus this variant appears to be widely expressed across species. Our failure to clone a C-1-1-like variant in mouse could reflect extremely low expression levels, expression in very selected tissues or specific developmental times, or simply absence of such variant in that species. We have not clarified yet which specific variant or variants are endogenously expressed in mouse embryo joints, whether variants expressed in the limbs are identical to those expressed in vertebral joints and tracheal rings, and whether specific variants are induced by GDF-5. Based on our in vitro data, it appears that each mammalian variant exerts similar biological activities in chondrocytes. Given such similar biological properties, it may actually not matter much whether specific variant(s) is/are

expressed in developing mammalian joints since each variant would be able to contribute to establishment of a permanent chondrocyte phenotype. This conclusion is somewhat at variance with observations and conclusions in our previous study in chick where we observed that C-1-1 is preferentially expressed in developing epiphyseal chondrocytes and has stronger stabilizing and anti-maturation properties than chERG when over-expressed (Iwamoto et al., 2000). One possible interpretation is that inclusion or exclusion of the 81 bp domain in chick ERG may have more profound effects due to chick-specific collateral contributions by other domains or subdomains. It may also be that given that chick expresses C-1-1 and chERG only, these two variants may need to have more distinct bioactivities and provide a range of functions needed in other tissues and stages. We should point out also that in developing chick long bones, C-1-1 and chERG are expressed not only in epiphyseal chondrocytes but also in the pre-hypertrophic zone of growth plate (albeit at different ratios), whereas ERG is largely if not exclusively restricted to epiphyseal chondrocytes in mouse. By exhibiting a more restricted gene expression pattern, mouse ERG may mostly be involved in behavior and developmental fate of epiphyseal chondrocytes and the apparent biological similarity of mouse ERG variants would still allow them to fulfill their predicted joint-associated function.

ERG and GDF-5

An interesting outcome of the present study is the evidence that GDF-5 is a rapid and effective inducer of ectopic ERG. Since ERG and GDF-5 exhibit virtually super-imposable spatio-temporal expression patterns, it is quite conceivable that GDF-5 may normally act to induce endogenous ERG. This is a likely and reasonable possibility given that GDF-5 is a very important regulator of synovial joint formation. Indeed, Kingsley and coworkers originally showed that joint defects seen in digits, wrist, ankle and other sites in the natural mouse mutant *brachypodism* are due to lack of GDF-5 (Storm et al., 1994). In a more recent study, the same group showed that compound ablation of GDF-5 and GDF-6 genes causes widespread joint defects and skeletal growth retardation (Settle et al., 2003). What has remained unclear, however, is the exact roles that GDFs and GDF-5 in particular have in joint formation. We may now surmise that an important and possibly critical function of GDF-5 would be to induce ERG and sustain the formation of permanent chondrocytes.

Admittedly, the above conclusion is a bit rudimentary when considered within the overall context of synovial joint formation, with its many steps and evens, its sheer complexity, and the many genes expressed (Archer et al., 2003; Pacifici et al., 2005). In addition to ERG, GDF-5 and GDF-6, developing joints express several other genes, including Wnt 14, Cux 1, Barx1, CD44, Chordin and Stanniocalcin (Guo et al., 2004; Hartmann and Tabin, 2001; Lizarraga et al., 2002; Stasko and Wagner, 2001). An intriguing feature is that some of these genes are chondrogenic and others are anti-chondrogenic. There have been several attempts to explain these opposing biological properties and how they would contribute to joint formation (Archer et al., 2003; Pacifici et al., 2005). An emerging view is that the anti-chondrogenic genes such as Wnt-14, Chordin and Cux 1, could be needed at early stages to define and maintain the mesenchymal character of the interzone. This would be particularly important for the central layer of the interzone that has pivotal functions, including cavitation and creation of a synovial space (Ito and Kida, 2000). On the other hand, the chondrogenic genes, including the GDFs, could act on interzone cells adjacent to the epiphyseal ends and favor their differentiation into chondrocytes. This would contribute to emergence of permanent articular chondrocytes. In this scenario, then, the ability of GDF-5 to trigger ERG expression would be quite important as it would allow newly-emerged chondrocytes to remain permanent, avoid their inclusion in the growth plate and maturation process, and establish the articulating ends of the long bones.

Growth plate and bone

The bulk of long bone cartilaginous anlagen is eventually replaced by bone tissue. This ossification process is closely related to the function of the growth plate and involves the initial formation of an intramembranous bone collar around the diaphysis followed by formation of endochondral bone and marrow replacing hypertrophic cartilage within the diaphysis (Olsen et al., 2000). It is quite well established that these ossification processes are closely dependent on, and tightly regulated by, the growth plate. Specifically, the bone collar is induced in conjunction with the pre-hypertrophic zone that produces the osteogenic factor Indian hedgehog (Koyama et al., 1996; Nakamura et al., 1997) and the endochondral bone requires functioning hypertrophic chondrocytes (Engsig et al., 2000; Komori et al., 1997; Koyama et al., 1999; Ueta et al., 2001). Given the above, it is reasonable that by lacking typical growth plates, the hERG3Δ81 transgenic mice would also exhibit considerable bone defects. As shown by our histochemical and μCT analyses, the trabecular bone/primary spngiosa within the diaphysis are particularly affected and largely absent, whereas the cortical bone appears to be less affected. One interpretation of these intriguing results is that formation of cortical bone may have been induced and sustained by Indian hedgehog that was still expressed in the transgenic mice, though at much reduced levels. Thus, defect in the diaphyseal trabecular bone/primary spngiosa region and the sharp transition we observe from cartilaginous tissue to intra-diaphyseal fibrotic tissue would be due to absence of functioning hypertrophic chondrocytes and lack of products the cells produce to permit and sustain endochondral ossification, including VEGF, MMP-9 and MMP-13 (Engsig et al., 2000; Vu and Werb, 2000).

Clinical relevance

Despite their durability and resilience, articular chondrocytes are prone to malfunction and even deterioration, and this is particularly evident in elderly individuals and osteoarthritic patients (Brandt, 2004). Several studies have shown that a significant number of osteoarthritic articular chondrocytes undergo a maturation-like process and express traits typical of growth plate cells, including an hypertrophic cell size, collagen X and APase activity (Kirsch et al., 2000; von der Mark et al., 1992). This progression toward maturation is inexorable and eventually leads to the complete disruption of articular cartilage and loss of joint function. We do not know yet whether this progression involves a loss of ERG expression or function, but this is certainly a plausible scenario. It is known that progression of chondrocyte maturation requires action by another transcription factor, Runx-2 (Komori et al., 1997). In a recent study, we directly compared the biological properties of Runx-2 and ERG in cultured chick chondrocytes (Iwamoto et al., 2005). We found that virally-driven Runx2-over-expression led to rapid chondrocyte maturation and a marked down-regulation of endogenous C-1-1 expression, while C-1-1 over-expression maintained the cells in an immature articular-like status without major rapid changes in endogenous Runx2 expression. The data suggest that when expressed at sufficiently high levels, each of these factors can have a dominant effect on the chondrocyte phenotype. It will thus be interesting and of medical relevance to establish in the future if ERG could be used as a therapeutic means to restore function and a permanent-like status on malfunctioning or aging articular chondrocytes. Given the biological potency of the mammalian variants shown here, this is a possibility well worth pursuing.

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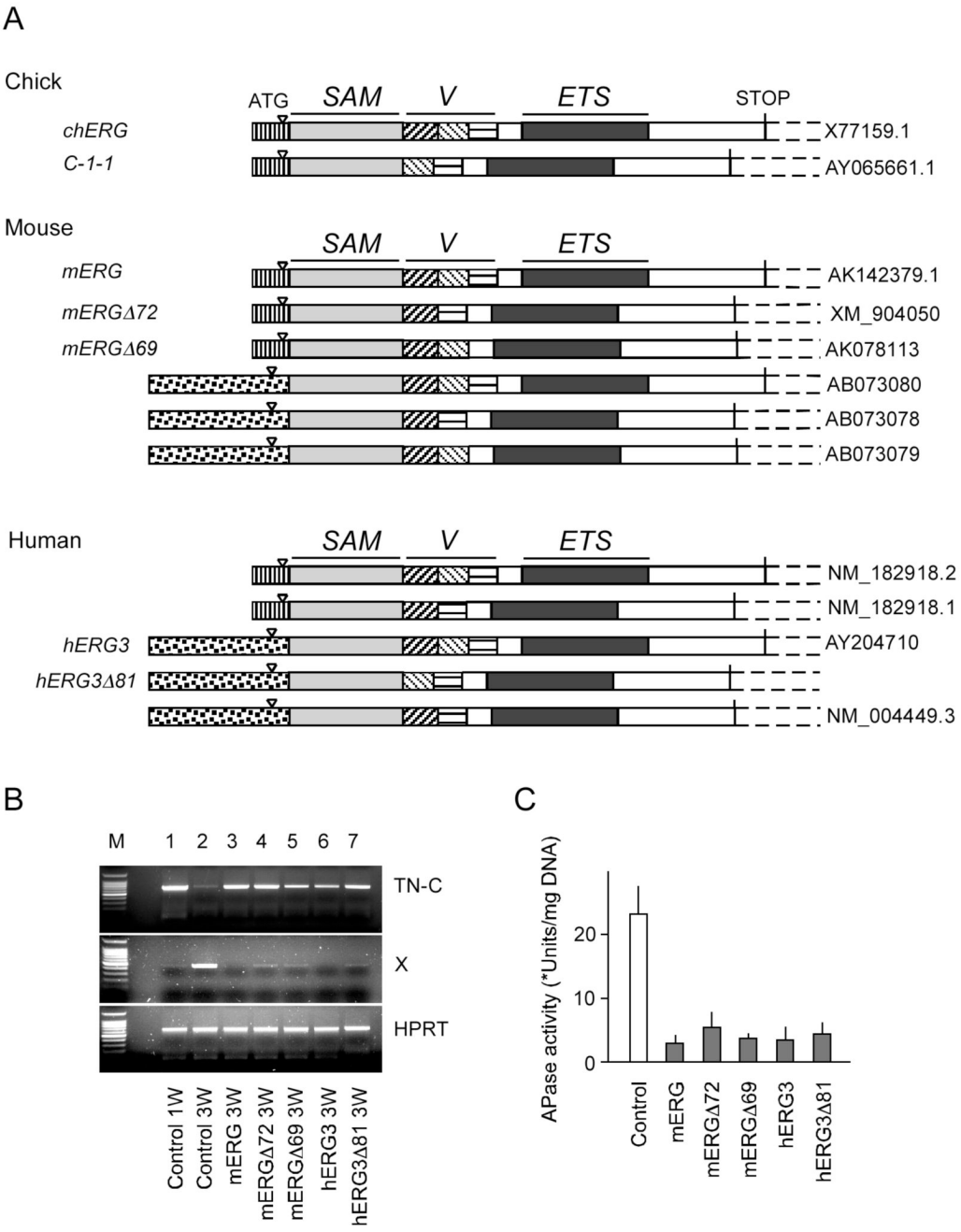

Fig. 1.

Schematic of chick, mouse and human ERG variants and analyses of biological activities. (A) Only major variants are shown in this scheme. Note the sterile alpha motif (SAM)/ Pointed domain; the variable region (V) containing 81, 72 and/or 69 bp exons; and the DNA binding ETS domain. (B) RT-PCR analysis of tenascin C (TN-C), collagen X (X) and HPRT expression in chick chondrocytes at 1 and 3 weeks of culture that over-expressed indicated mouse or human ERG variants (lanes 3-7). Insert-less virus was used to infect control cells (lanes 1-2). (C) APase activity in 3 week-old chondrocyte cultures infected with control insert-less virus or viruses encoding indicated mouse or human ERG variants. APase activity was normalized to DNA contents and is expressed as the average of three independent cultures ± S.D.

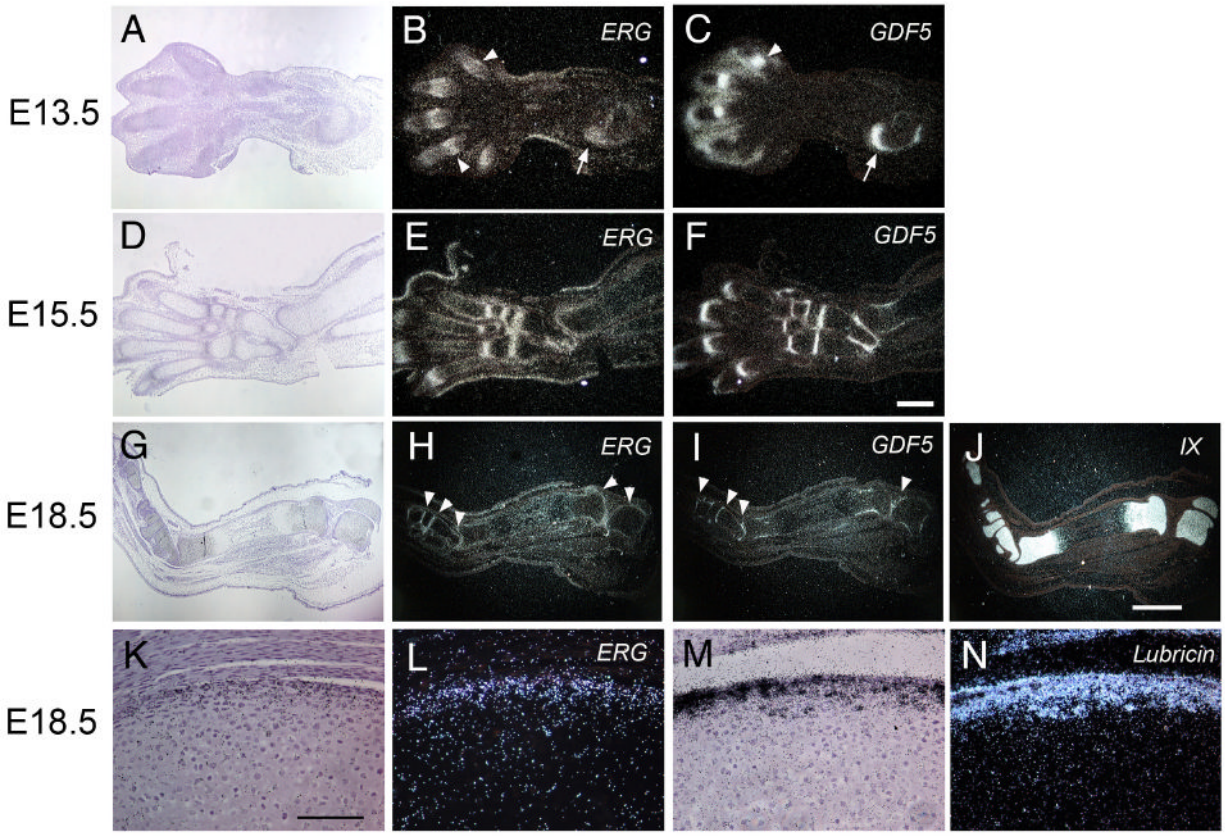

Fig. 2.

ERG is expressed in developing mouse limb joints. Serial sections of hind limbs from E13.5 (A-C), E15.5 (D-F) and E 18.5 (G-N) mouse embryos were hybridized with radio-labeled riboprobes encoding ERG, GDF-5, collagen IX or lubricin/PRG4. Note at E13.5 and E15.5 (A-F) that ERG is expressed in nascent joints and its expression is quite similar to that of GDF-5. By E18.5, ERG expression is lower and restricted to most-epiphyseal articular chondrocytes (H and L) that express also lubricin/PRG4 (M-N). Bar for A-J, 1 mm; and bar for K-N, 100 μm.

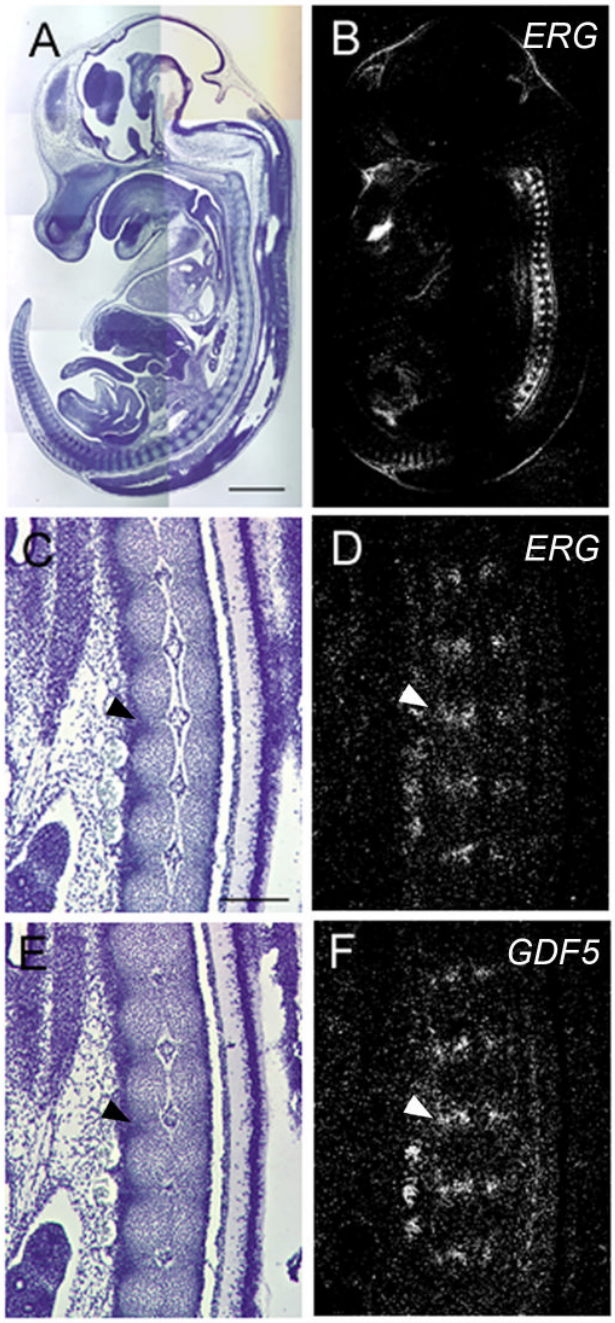

Fig.3.

ERG and GDF-5 are co-expressed in developing intervertebral discs. Serial sagittal sections of E15.5 mouse embryos were hybridized with ERG (A-D) or GDF-5 (E-F) riboprobes and examined at low (A-B) and high (C-F) magnification. Arrowheads point to the strong ERG and GDF-5 hybridization signal visible in the annulus fibrosus in each disc. Bar for A-B, 1 mm; and bar for C-F, 200 μm.

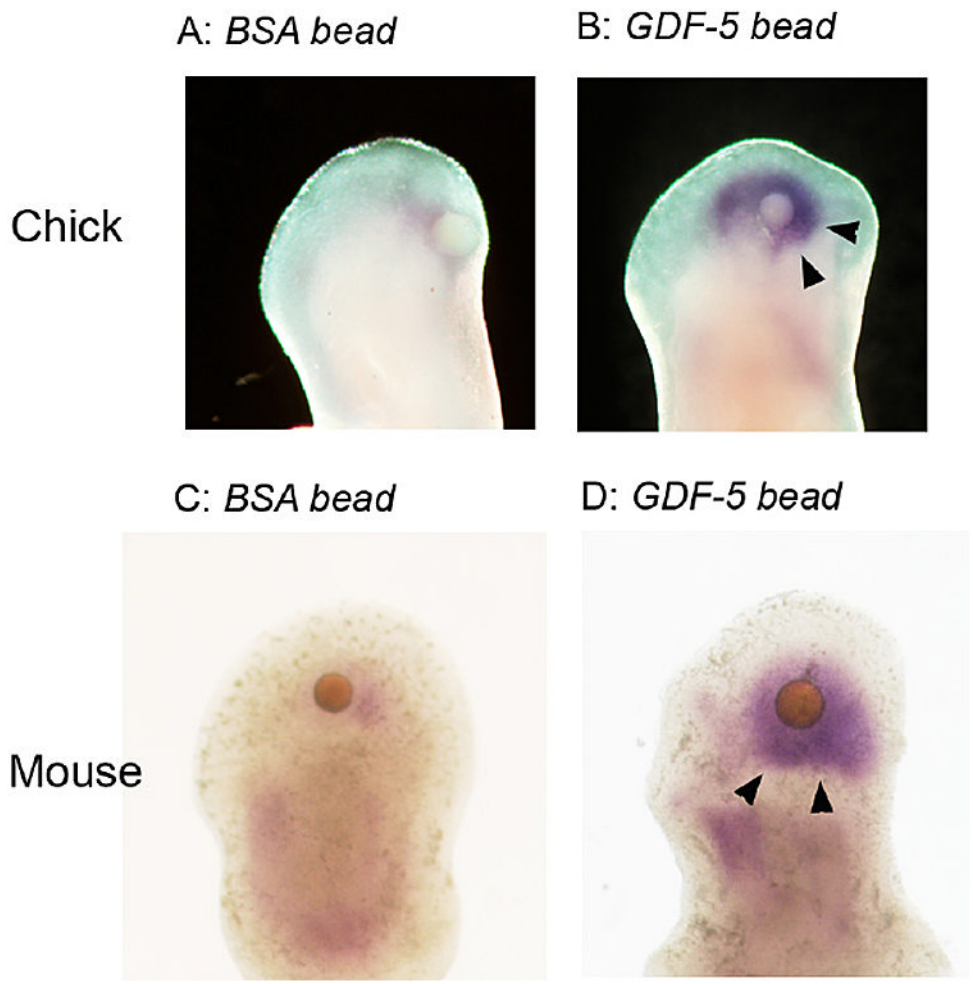

Fig.4.

GDF-5 induces ectopic ERG expression. Day 5.5 chick embryo and E12.5 mouse embryo autopods were placed in organ culture and implanted microsurgically with a single Sepharose-heparin bead pre-soaked with BSA or GDF-5 (10 μg/ml). Specimens were maintained for 6 hrs and then processed for whole mount hybridization for ERG expression. Positive ERG signal is purple and can be seen surrounding each GDF-5 bead (B and D, arrowheads) but not around control BSA beads (A and C).

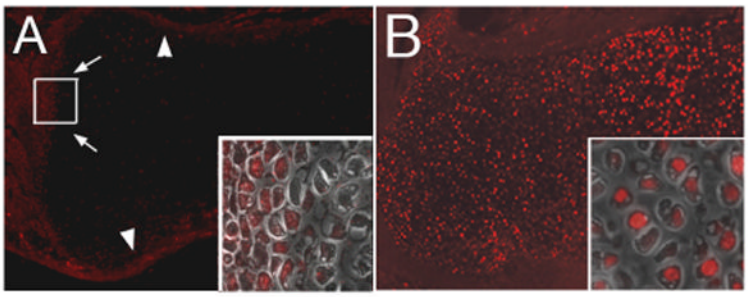

Fig.5.

ERG is widespread in transgenic cartilage. Tibias from E18.5 wild type and hERGΔ81 transgenic embryos were processed for ERG immunostaining, using antibodies that recognize each variant. Note in (A) that ERG is most appreciable at the epiphyseal ends in wild type tissue (arrows) and peri-epiphyseal perichondrium (arrowheads), whereas it is widespread throughout the transgenic cartilage tissue (B). Inset in (A) is a higher magnification view of boxed area showing that ERG is largely nuclear. Inset in (B) shows that many but not all transgenic chondrocytes contain nuclear ERG, a reflection of the likely mosaicism of the transgenic mice.

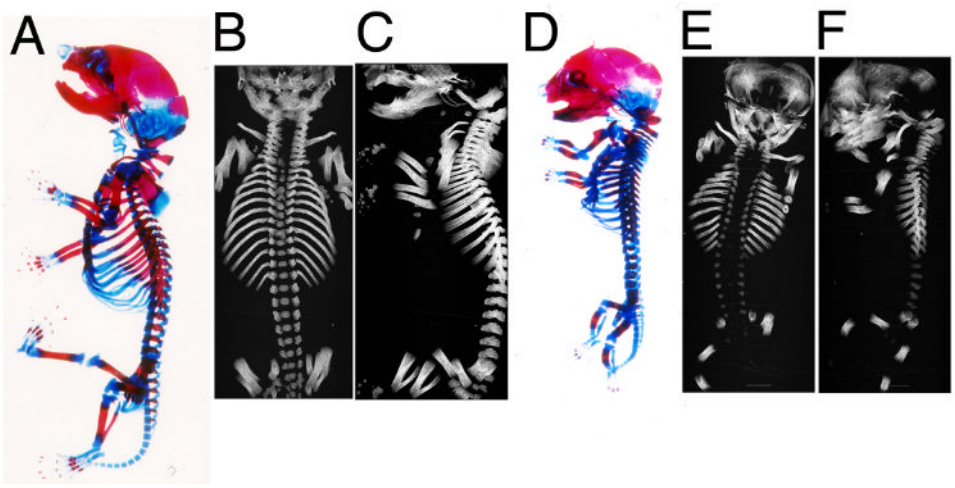

Fig. 6.

Transgenic hERGΔ81 expression in cartilage leads to dwarfism. Skeletons of E18.5 wild type (A-C) and transgenic (D-F) mouse embryos were processed for staining with alizarin red and alcian blue (A and D) or subjected to soft x-ray analysis (B-C and E-F). Note in (A) and (D) that the transgenic mice are much smaller than wild type littermates and exhibit higher overall amounts of cartilaginous tissue and lower amounts of alizarin red-staining tissue. Note also that the transgenic mice are hypo-mineralized (E-F) compared to control (B-C).

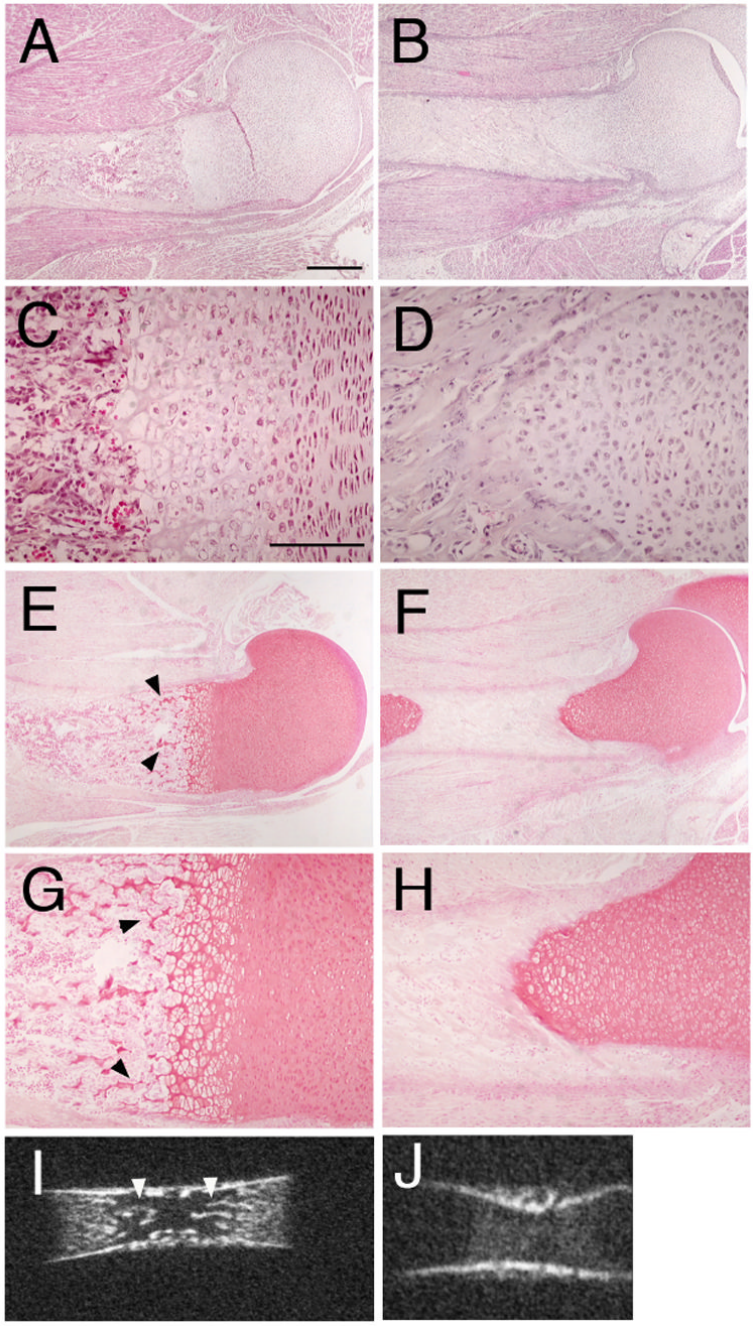

Fig. 7.

Growth plate organization and ossification patterns are abnormal in transgenic mice. Humeri from E18.5 transgenic embryos and wild type littermates were processed for histology (A-D), histochemical detection of proteoglycan matrix by Safranin O staining (E-H) and μCT analysis (I-J). Transgenic cartilaginous elements lack a growth plate organization (B and D) that is readily recognizable in wild type (A and C). Note also that there is a typical gradual transition from hypertrophic cartilage to bone and presence of primary spongiosa in wild type (E, G and I, arrowheads), whereas there is a sharp boundary, no gradual transition and minimal primary spongiosa in transgenic tissue (F, H and J).

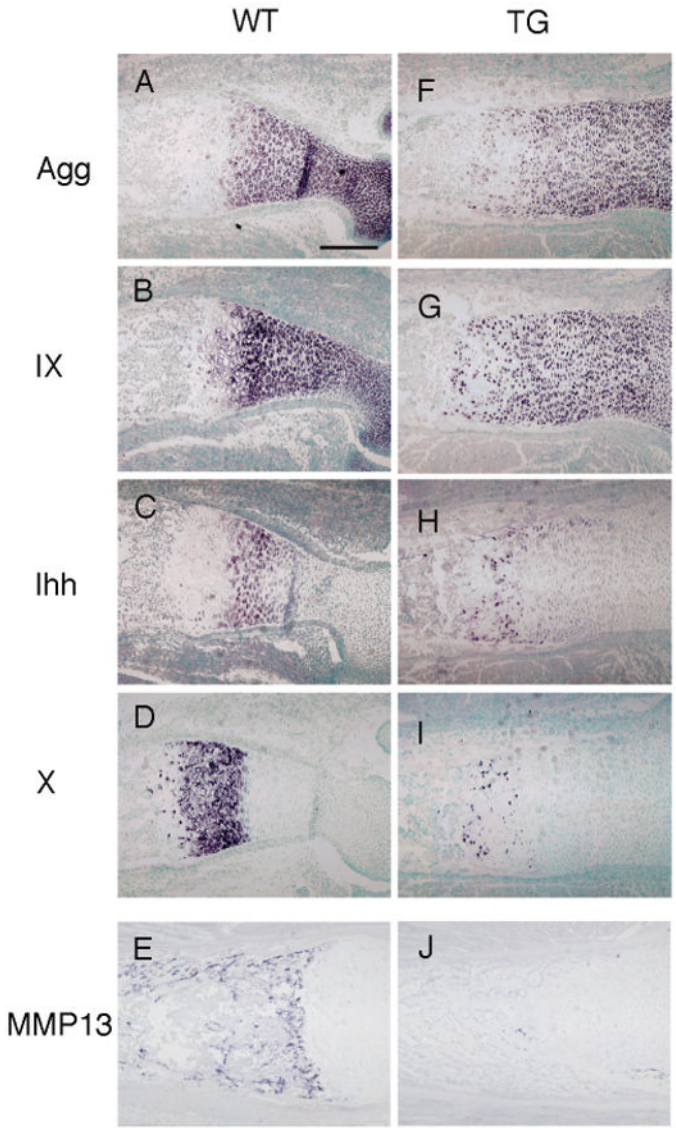

Fig. 8.

Gene expression of maturation markers is markedly reduced in transgenic growth plates. Sections from the distal half of E18.5 wild type (A-E) and transgenic (F-J) humeri were hybridized with digoxigenin-labeled cDNA probes encoding: aggrecan (A,F); collagen IX (B,G); Indian hedgehog (C,H); collagen X (D,I); and MMP13 (E,J). Note that aggrecan and collagen IX expression are equally strong in wild type and transgenic cartilage, whereas expression of maturation markers is markedly lower in transgenic tissue. Sections in (A-D) and (F-I) are serial sections, whereas those in (E) and (J) are from other tissue blocks. Bar, 100 μm.

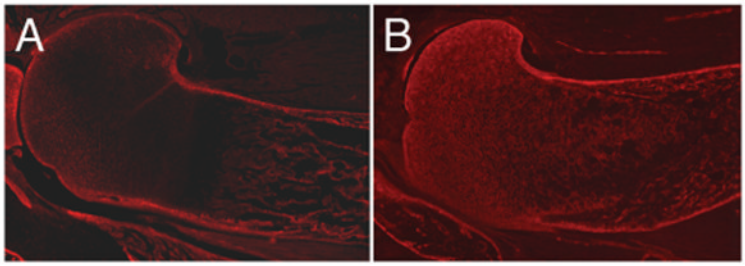

Fig. 9.

Tenascin-C is widespread in transgenic cartilage. Sections from E18.5 wild type (A) and transgenic (B) tibias were de-masked and processed for immunostaining with tenascin-C antibodies. Note that the protein is limited to the epiphyseal end in wild type long bone anlaga as expected (A), whereas it is present throughout the transgenic cartilage (B).