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Erg cooperates with TGF- β to control mesenchymal differentiation

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Abstract

Transforming growth factor β (TGF- β) signaling plays an integral role in skeletal development. Conditional deletion of the TGF- β type II receptor (Tgfr2) from Type II Collagen (Col2a) expressing cells results in defects in development of the annulus fibrosus (AF) of the intervertebral disc (IVD). We previously used microarray analysis to search for marker genes of AF as well as transcription factors regulated by TGF- β during AF development. The transcription factor avian erythroblastosis virus E-26 (v-ets) oncogene related (*Erg*) was identified in the microarray screen as a candidate regulator of AF development. To study the effects of TGF- β on AF differentiation and the role of *Erg* in this process, we used mouse sclerotome grown in micromass cultures. At 0.5 ng TGF- β /ml, sclerotome cells started to express markers of AF. Regulation of *Erg* by TGF- β was confirmed in these cells. In addition, TGF- β soaked Affi-gel beads implanted into the axial skeleton of stage HH 25 chick embryos showed that TGF- β could induce expression of *Erg* mRNA in vivo. Next, an adenovirus to over-express *Erg* in primary sclerotome micromass cultures was generated. Over-expression of *Erg* led to a change in cell morphology and inhibition of differentiation into hyaline cartilage as seen by reduced Alcian blue staining and decreased *Sox9* and *c-Maf* expression. *Erg* was not sufficient to induce expression of AF markers and expression of *Sca1*, a marker of pluripotent progenitor cells, was upregulated in *Erg* expressing cells. When cells that ectopically expressed *Erg* were treated with TGF- β , enhanced expression of specific differentiation markers was observed suggesting *Erg* can cooperate with TGF- β to regulate differentiation of the sclerotome. Furthermore, we showed using co-immunoprecipitation that *Erg* and Smad3 bind to each other suggesting a mechanism for their functional interaction.

Keywords

intervertebral disc; annulus fibrosus; sclerotome; Smad3; Adamtsl2; Scleraxis

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Introduction

Intervertebral disc (IVD) are derived from embryonic structures called the sclerotome and notochord [1–3]. The nucleus pulposus, the cushioning core of the mature IVD, is derived from the notochord while the annulus fibrosus (AF), which provides the structural properties of the IVD, is derived from sclerotome. The sclerotome is derived from somites, transient structures that determine the segmented nature of the embryo [4]. In response to signals from the notochord and floor plate of the neural tube, the maturing somites undergo dorsal-ventral compartmentalization establishing the dermomyotome and sclerotome, the latter forming most of the connective tissues of the future axial skeleton. The sclerotome is a pluripotent mesenchyme and gives rise to the cartilage of the vertebral bodies, AF of the disc, and tendon. Each sclerotome segment demonstrates rostral to caudal polarity and due to resegmentation of sclerotome, the caudal domain of the sclerotome will form the anterior structures of each vertebrae and the rostral domain forms the posterior structures [5, 6]. The AF will form from the cells in the sclerotome adjacent to the border between the sclerotome halves. While many aspects of development in the axial skeleton have been elucidated, the development of the IVD is still poorly understood. Transforming growth factor-beta 3 (Tgfb3) is one of the earliest markers of the developing IVD within the sclerotome [7].

TGF- β is a critical component for many aspects of development [8]. TGF- β s signal through heteromeric Type I and Type II serine/threonine kinase receptors. The current model is that TGF- β binds to the Type II receptor (Tgfb2) on the cell surface, recruits the type I receptor (Tgfb1) to form a heterotetrameric complex, then Tgfb2 phosphorylates the type I receptor activating the type I serine/threonine kinase [9]. Smad proteins are phosphorylated by the type I receptor, translocate to the nucleus and act as transcription factors [10]. In particular, TGF- β signaling is necessary for proper development of the AF of the IVD. Conditional deletion of *Tgfb2*, results in the disruption of development in the axial skeleton, including loss of the AF without affecting the nucleus pulposus of the IVD [11, 12]. Previously, we used microarray analysis to identify genes that could be used as markers of the AF during development, as well as genes downstream of TGF- β signaling that would potentially be involved in controlling differentiation of the AF [13]. We identified avian erythroblastosis virus E-26 (v-ets) oncogene related (*Erg*) as being localized to the IVD during development, down-regulated by *Tgfb2* deletion in vivo, and up-regulated by TGF- β treatment in sclerotome cultures suggesting that it may play an important role in differentiation of the AF from sclerotome.

Erg is a member of the ERG sub group of the ETS family of transcription factors [14]. These transcriptions factors are all grouped based on their ETS binding site and have been shown to frequently act as co-transcription factors.[15, 16] It has also been shown that ERG can activate a TGF- β response element [17] and that the common fusion proteins seen in prostate cancer can act as dominant negative forms and inhibit expression of *Tgfb2* [17]. During development *Erg* has been shown to be expressed in the developing IVD as well as the interzone in developing synovial joints [18–23]. A chondrocyte specific form of ERG, known as ERG C-1-1, was identified in chick and when over expressed blocked hypertrophic differentiation in chondrocytes [20–22]. The role of *Erg* in development of the

AF was not addressed. We therefore evaluated Erg for its potential to regulate AF differentiation.

In this study, we confirm that TGF- β regulates expression of Erg in sclerotome cultures and in vivo. We used adenovirus vectors to over-express Erg in sclerotome and show that Erg blocks chondrogenesis in the cultures but is not sufficient to induce expression of markers for AF differentiation. Expression of *Sca1*, a marker of pluripotent progenitor cells, is up-regulated in Erg expressing cells suggesting that Erg maintains cells in a pluripotent state. We then show that Erg can cooperate with TGF- β to stimulate expression of *Adamts2* and *Scx*, markers of AF and tendon respectively. Furthermore, Erg protein associates with Smad3 in sclerotome cells. We propose that Erg cooperates with TGF- β in the development of the IVD, promoting AF gene expression and preventing the formation of vertebral cartilage in the IVD space.

Methods

Sclerotome Cell Isolation and Culture

Cells were isolated from wild type (Bl/6 X ICR) e11.5 mouse embryos as described in [13]. Cells were plated in 20 μ l drops at a density of 1.5×10^7 cells/ml. Drops were allowed to set for one hour before flooding the well with media. Cells were treated with TGF β 1 or BMP the following morning. For experiments with adenovirus, cells were resuspended in media containing 50 MOI adenovirus before being placed in micromass culture. TGF- β 1 or BMP4 treatments were performed 48 hours after transduction. Cells were fixed with 4% PFA then stained with 1% alcian blue in 0.1N HCl 3 or 7 days post transduction. All animal use was approved by the University of Alabama at Birmingham's Institutional Animal Care and Use Committee.

Adenovirus production and transduction

Full-length mouse *Erg* was cloned into pEC3.1 which contains a CMV promoter and an IRES-*Gfp* flanked by attL sites. This vector was then recombined using the Gateway Cloning system into pAdBlockIt (Invitrogen). Virus was produced in HEK 293A cells (Invitrogen) following the manufacturer's protocol. Virus was concentrated by pelleting the cells and resuspending in 1 ml of media before freeze thawing according to the manufacturer's protocol. Virus was titered by infecting 293A cells with serial dilutions of viral supernatant. After 48 hours, the number of GFP-positive cells was counted and concentration calculated based on dilution. Control virus contained an additional *Gfp* in place of *Erg*.

Chick Bead Implants

Specific pathogen free, white leghorn fertilized chicken eggs were obtained from Charles River Labs and incubated at 39°C. Embryos were staged according to Hamburger and Hamilton [24]. AffiGel Blue beads 100–200 mesh (Bio-Rad) were soaked in 10 ng/ μ l porcine TGF-B (RD Systems) or 10 ng/ μ l BSA for one hour. The beads were then implanted in forelimb and lower spine of HH 23–25 chick embryos. Chick embryos were harvested after 24 hours for in situ hybridization.

In Situ Hybridization

Whole mount in situ hybridization was performed on stage HH 27 chick embryos 24 hours after implantation. Embryos were fixed in 4% PFA. Embryos were then stored in 100% methanol at -20°C . The probe for the chicken *Erg* was a kind gift from Masahiro Iwamoto [20] and was labeled with digoxigenin. In situ hybridization was performed as described in Riddle et al [25]. BM Purple (Roche) was used to visualize alkaline phosphatase label.

qRT-PCR

RNA samples were collected from sclerotome micromass cultures, using RNeasy Plus (QIAGEN). Real time PCR was done using QuantiFast SYBR Green RT-PCR Kit (QIAGEN) run on a Lightcycler 480 (Roche). Primers were designed using NCBI primer BLAST or selected from Primer Bank [26, 27] (Table 1). Differential expression and statistical significance were analyzed using REST 2009 software (QIAGEN) which allows for efficiency correction as well as rigorous statistical testing of non-parametric data [28]. REST uses a pair-wise fixed reallocation randomization test to determine significance. HPRT was used as the normalization control in all experiments. Expression was determined relative to the untreated or Ad-GFP infected, untreated controls as indicated.

Western Blot, Immunoprecipitation-Western Blot Analysis

For western blots, 10ug of protein was run on a 10% SDS-PAGE gel then transferred to a PVDF membrane. ERG was detected with anti-ERG (Abcam #77258) 1:1000 and GAPDH (Santa Cruz # sc-20357) was used as a loading control.

For immunoprecipitation (IP)-western blot assays, protein samples were collected using lysis buffer (20mM Tris pH8.0, 150mM NaCl, 2mM EDTA pH8.0, 10% Glycerol) with 1% TritonX-100 and processed according to standard protocols. Since *Erg*, Smad3 and IgG heavy chain are all close to 50kDa, we used antibodies that were made in different species for the immunoprecipitation and the immunoblot to minimize the detection of the IgG heavy chain. Goat anti-ERG (Abcam #77258), Rabbit anti-ERG (Abcam # 92513), and Rabbit anti-Smad3 (Cell Signaling #9513) antibodies were used as indicated. Secondary antibodies used were HRP conjugated anti Rabbit IgG (Santa Cruz #B2213) and HRP conjugated anti Goat IgG (Santa Cruz #G0208).

Results

TGF- β induces expression of *Erg*, an AF enriched transcription factor

Primary sclerotome cells grown in micromass in the absence of growth factors demonstrate formation of Alcian Blue stained nodules containing round cells representing hyaline cartilage, similar to what is seen in cultures of limb mesenchyme (Figure 1A) [29]. We previously showed that sclerotome cultures treated with 5ng TGF- β /ml demonstrated a reduction in Alcian blue stained nodules. In addition, the cells in the culture were not round and took on a spindle shape [13]. Others have recently observed a similar response to TGF- β in limb mesenchyme grown in micromass culture [29–31]. To further characterize the effects of TGF- β on sclerotome micromass cultures we treated cells with varying concentrations of TGF- β 1 or BMP4 (Figure 1A). BMP is a known chondrogenic factor [32]

and treatment with BMP resulted in a dose dependent increase in Alcian blue stained nodules with hyaline cartilage morphology (Figure 1A). Treatment with varying doses of TGF- β resulted in a dose dependent decrease in Alcian blue stained nodules (Figure 1A). We previously showed an increase in markers for AF in sclerotome treated with 5ng TGF- β /ml [13]. Here we confirm up-regulation of Fibromodulin (Fmod) and Adamtsl2 by varying doses of TGF- β in sclerotome cultures (n=4 separate experiments) using quantitative real time RT-PCR (Figure 1B) where expression was determined relative to untreated controls and HPRT was used as the normalization control. Adamtsl2 was regulated in a dose-dependent manner between 0.5 and 5 ng TGF- β /ml however, maximal expression of Fmod was observed at the lowest dose tested. Scleraxis (Scx), a marker of tendon differentiation was also up-regulated in a dose dependent manner. It was previously shown that TGF- β regulates tendon differentiation in culture and in vivo [33]. Sox9, one of the master regulators of cartilage differentiation, was not regulated by TGF- β at any dose tested whereas, c-Maf, a transcriptional partner of Sox 9 [34] was down regulated by TGF- β (Figure 1C). Sca1, a marker of pluripotent mesenchymal stem cells [35], was not regulated by TGF- β (Figure 1C). The results confirm previous studies indicating TGF- β promotes a fibrous phenotype in limb mesenchymal cells and extend the studies to sclerotome [13, 29, 31].

Using microarray screens, we previously identified Erg as a transcription factor that was enriched in the AF and regulated in sclerotome by TGF- β [13]. To verify regulation of Erg by TGF- β we measured Erg mRNA levels in sclerotome cultures treated with 5ng TGF- β /ml for 24 hours (Figure 2A; n= 7 separate experiments). TGF- β treatment resulted in a significant increase in Erg mRNA levels in the sclerotome cultures. To determine if TGF- β could regulate Erg in an in vivo model system, we implanted TGF- β soaked Affy-blue beads in chicken embryos in ovo and looked for induction of *Erg* mRNA by whole mount in situ hybridization. Beads soaked in bovine serum albumin (BSA) were used as controls. After 24hrs with TGF- β , we saw increased *Erg* mRNA expression in both the limb bud (Figure 2 B,C, arrows) and spine (Figure 2 D–F, arrows) compared to the BSA controls. The results indicate that TGF- β regulates expression of Erg in both cell culture and in vivo models.

ERG is not sufficient to induce expression of AF marker genes

Since Erg was localized to the AF and regulated by TGF- β , we tested the hypothesis that Erg, like TGF- β , would regulate expression of markers for AF in sclerotome cells. To test this hypothesis, we generated an adenovirus vector that would express mouse Erg (Ad-Erg) when transduced into sclerotome cells in culture. An adenovirus expressing only GFP (Ad-GFP) was used as a control. Cells were infected with Ad-GFP or Ad-Erg for 48 hours at which time RNA and protein were isolated from the cells to confirm increased expression of Erg by real time RT-PCR and western blot (Figure 3 A, B). Both mRNA expression (Figure 3 A) and protein levels (Figure 3 B) of Erg were dramatically increased by transduction with ad-Erg. Overexpression of Erg in mouse sclerotome micromass cultures for one week resulted in inhibition of Alcian blue staining (Figure 3 C, D). Very few chondrocytic nodules were observed. The Erg cells appeared spindle shaped. The loss of cartilage nodules and spindle shaped cells could also be seen using phase contrast microscopy (Figure 3 E, F) and with Toluidine blue O staining of sections through the cultures (Figure 3 G, H).

The loss of Alcian blue stained cartilage nodules and spindle shaped cells lead us to hypothesize that Erg could promote AF differentiation in the sclerotome cultures. RNA isolated from Ad-GFP and Ad-Erg infected cells was used in real time RT-PCR to measure the expression of hyaline cartilage and AF enriched genes (Figure 4). Gene expression relative to the Ad-GFP infected, untreated controls was determined. HPRT was used as a normalization control. Consistent with the reduction in Alcian blue stained cartilage nodules observed, expression of Erg resulted in down-regulation of Sox9 and c-Maf, markers of chondrocyte differentiation [36]. Nevertheless, Erg was not sufficient to stimulate expression of Fmod or Adamts12, markers of AF, or Scx, a marker for tendon differentiation (Figure 4). Likewise, we did not detect changes in expression of smooth muscle actin, which would indicate smooth muscle differentiation, or *Pecam*, a marker for endothelial cell differentiation (data not shown). However, increased expression of *Sca1*, a marker of pluripotent mesenchymal stem cells [35], was observed (Figure 4). The results suggested that in the absence of other factors Erg may act to maintain the mesenchymal cells in a pluripotent state.

Erg and TGF- β cooperate to regulate expression of specific genes

Since Erg appeared to maintain cells in a pluripotent state, we tested the hypothesis that this would make the cells more competent to respond to TGF- β . To this end we infected cells with either the Ad-GFP or Ad-Erg virus and then treated the cells with varying concentrations of TGF- β . Cells infected with the control Ad-GFP virus and treated with TGF- β demonstrated a dose dependent decrease in Alcian blue stained nodules as previously shown (Data not shown). Expression of specific marker genes relative to untreated, Ad-GFP infected cells (reference set to 1.0) was compared in Ad-GFP infected cells treated with 1ng TGF- β /ml, untreated Ad-Erg infected cells, and Ad-Erg infected cells treated with 1ng TGF- β /ml for 24 hours (Figure 5A,B; n= 4 separate experiments). HPRT was used as a normalization control. Markers of AF, Fmod and Adamts12, were up-regulated by TGF- β and unaffected by Ad-Erg alone, as expected. However, expression of Ad-Erg and TGF- β treatment together resulted in a significant increase in Adamts12 expression above that observed with TGF- β treatment alone suggesting cooperation between Erg and TGF- β signaling in the regulation of *Adamts2* (Figure 5A). In contrast, Erg did not appear to contribute to regulation of Fmod mRNA (Figure 5A). Similar to *Adamts2*, Erg and TGF- β also appeared to cooperate to regulate the expression of the tendon marker, *Scx* (Figure 5A). Likewise, down-regulation of c-Maf mRNA was significantly greater in Ad-Erg infected cells treated with TGF- β than in untreated Ad-Erg infected cells suggesting Erg and TGF- β cooperate to down-regulate c-Maf mRNA (Figure 5B). Sox9 mRNA levels were not regulated by TGF- β and down-regulated by Ad-Erg as we showed previously. Sox9 was still significantly down regulated in TGF- β treated Ad-Erg expressing cells although to a lesser extent than with Ad-Erg alone (Figure 5B). *Sca1*, which is regulated by Ad-Erg but not by TGF- β was still up-regulated in TGF- β treated Ad-Erg infected cells although to a lesser degree, possibly due to differentiation induced with TGF- β treatment (Figure 5B). Together the results suggest that TGF- β and Erg cooperate to regulate specific markers for AF and tendon differentiation.

Since Smad3 is the primary signaling protein for TGF- β , acting as a transcription factor when activated after ligand-receptor binding, we next tested the hypothesis that Erg and Smad3 could participate in a protein complex that would potentially mediate the functional cooperation observed. To test this hypothesis, immunoprecipitation followed by western blot was used (Figure 6). First, primary sclerotome cells were infected with Ad-Erg or Ad-GFP virus. Smad3 was immunoprecipitated from protein lysates generated from the infected cells. Immuno-complexes were resolved on an SDS-polyacrylamide gel and the proteins were transferred to nitrocellulose membranes, which were incubated with antibody directed to the Erg protein followed by a secondary antibody for detection. A large amount of the ectopic Erg protein was observed in the immunocomplexes that were pulled down with the Smad3 antibody indicating that Smad3 can form a complex with Erg (Figure 6A). Expression of Ad-Erg was confirmed by western blot using an aliquot of protein samples before immunoprecipitation (Figure 6A, input). Complexes of endogenous Erg and Smad3 could also be pulled down with the anti-Erg antibody (Figure 6B). The results indicate that Smad3 and Erg can participate in the formation of protein complexes.

Discussion

Having previously shown that TGF- β signaling pathways play a role in IVD development [12], we focused on transcription factors regulated by TGF- β as potential mediators of AF differentiation. From an extensive list of genes expressed in the AF during development, we focused on Erg, a transcription factor that is known to be involved in skeletal development [20–22]. In sclerotome cell cultures TGF- β is able to increase the expression of Erg, along with other markers of the AF. The localization of Erg to the AF during development and its regulation by TGF- β led us to investigate its role in controlling differentiation of AF from mesenchymal progenitor cells. Our original hypothesis was that Erg acts as a down-stream target of TGF- β in controlling AF differentiation.

The effect of ectopic expression of Erg in sclerotome cells was partially similar to that of TGF- β treatment in that both inhibited formation of Alcian blue stained hyaline cartilage nodules. Inhibition of *Sox9* and *c-Maf* expression by Ad-Erg corresponded with inhibition of formation of chondrocytic nodules and indicated that the cells were not differentiating along the path of hyaline cartilage towards mineralization, which is the default for these cultures. Nevertheless, none of the genes we analyzed as markers of AF or tendon differentiation were up-regulated in response to Erg alone. This led us to consider other mesenchymal differentiation pathways including differentiation into adipocytes, smooth muscle, or endothelial cells, which would have resulted in accumulation of lipids within the cells, expression of α SMA, or *Pecam* respectively. Since Erg was previously shown to be involved in vascular development and global deletion of Erg results in early embryonic lethality due to severe hemorrhaging [37] it was important for us to exclude differentiation into either smooth muscle or endothelial cells. We did not observe changes in expression of any of these relevant markers in Ad-Erg infected cells. Since these mesenchymal derivatives were excluded, we were left with the possibility that the cells were remaining in an undifferentiated state.

Erg expression resulted in increased levels of Sca1 mRNA, a known marker for pluripotency in mouse [35], suggesting Erg may help to maintain the cells in an undifferentiated state until they can respond to the appropriate growth factors during development. In hematopoietic cells, it has been shown that Erg is involved in maintaining the stem cell population [38]. This suggested the possibility that Erg is involved in maintaining a pool of undifferentiated cells in the sclerotome during development. The cells would then be primed to receive an instructive signal from TGF- β to drive differentiation to AF. Knowing that Erg like other ETS family members is capable of acting as a co-transcription factor, we looked for effects of Erg on TGF- β signaling in the sclerotome cells. The results indicated that Erg cooperates with TGF- β to enhance the expression of Adamtsl2 and Scx mRNA. Although Fmod is a marker of the AF and is regulated by TGF- β , Erg did not affect expression of Fmod either alone or in combination with TGF- β . It is possible that for Fmod we were not looking at the right dose range of TGF- β to cooperate with Erg or, more likely, Erg only modulates expression of a subset of TGF- β responsive genes. Cooperation between TGF- β and Erg to regulate Adamtsl2, Scx, and c-Maf raise the possibility that Erg could interact directly with Smad3 to promote or repress transcription of specific genes. Previous reports indicating that Erg can stimulate a common TGF- β reporter element support this hypothesis [17]. Here we show through immunoprecipitation followed by western blot that Erg and Smad3 are present together in protein complexes in primary sclerotome cells. We did not test the interaction of Erg with other Smads including the BMP associated Smads 1/5/8 so it is possible that Erg could interact with other Smads. In addition, at this time we do not know if or how TGF- β modulates the interaction between Erg and Smad3.

Although Erg appears to block formation of hyaline cartilage in sclerotome cultures it is not sufficient to induce differentiation of mesenchymal cells on its own. This suggests that blocking the default chondrogenic pathway is not sufficient to induce differentiation along another lineage and an instructive signal, like TGF- β , is also required. In addition to instructive signals that promote AF differentiation, an environment permissive for AF differentiation is critical during IVD development. It is possible that Erg mediated inhibition of chondrogenesis in the disc space provides this permissive environment. Early in development of the axial skeleton, BMP is synthesized by cells that will form the IVD. BMP protein is then transported and concentrated in the area where the vertebral body will form [39, 40]. To prevent chondrogenesis and subsequent endochondral bone formation in the IVD space, it is necessary to block the action of BMP in this area. Multiple BMP inhibitors are synthesized and localized to the developing IVD including Noggin and Chordin [8]. It has been shown in other model systems that TGF- β can antagonize BMP signaling although the mechanism is not known [41]. It is possible that Erg could act as an additional method to prevent BMP mediated chondrogenesis in the IVD space.

In summary, Erg is expressed in the AF and its expression is regulated by TGF- β . Ectopic expression of Erg is not sufficient to induce AF differentiation; however, TGF- β and Erg act synergistically to regulate Adamtsl2, a marker of AF. We propose that Erg modulates TGF- β activity during development of the IVD.

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Highlights

- TGF- β stimulates expression of markers for AF in sclerotome cultures.
- Erg expression is regulated by TGF- β .
- Ectopic expression of Erg is not sufficient to induce expression of AF markers.
- TGF- β and Erg act synergistically to regulate Adamtsl2, a marker of AF.
- Erg protein interacts with Smad3 in sclerotome cells.

Figure 1. TGF- β and BMP4 act in a dose-dependent fashion to regulate differentiation in sclerotome cells

(A) Alcian blue staining of micromass cultures treated with varying doses of TGF- β 1 or BMP4 for 5 days. (B, C) Quantitative real-time RT PCR of AF enriched genes up-regulated by TGF- β 1 (B) and genes down regulated or unchanged (C) by TGF- β 1 treatment. Data analyzed by REST. Expression is relative to untreated cells, which are set at 1.0 (dashed line). HPRT was used as the normalization control. *** with a horizontal line indicates $p < 0.05$ relative to untreated controls. The step lines indicate $p < 0.05$ between the two samples connected. $n = 4$.

Figure 2. TGF- β stimulates Erg expression

(A) Quantitative real-time RT PCR showing *Erg* expression in sclerotome micromass cultures after treatment with 5ng TGF- β 1/ml for 24 hours. Data analyzed by REST. Expression is relative to untreated cells set at 1.0. HPRT was used as the normalization control. * indicates $p < 0.05$. $n = 7$. (B–F) In situ hybridization for *Erg* 24 hours post implantation of TGF- β 1 (C, E, F) or BSA (B, D) soaked bead in the forelimb (B, C) or lower spine (D–F) of HH25 stage chick embryos. Arrows denote ectopic Erg expression. Asterisks indicate location of the bead.

Figure 3. ERG inhibits chondrogenesis in sclerotome cells

(A) Quantitative real-time RT PCR was used to verify over-expression of Erg mRNA 3 days post-transduction. Two days of TGF- β treatment did not further regulate Erg mRNA levels in Ad-Erg infected cells. Expression relative to untreated Ad-GFP transduced cells is shown (set to 1.0, dashed line). RT-PCR data was analyzed using REST. HPRT was used as the normalization control. * indicates $p < 0.05$. $n = 7$. (B) Western blot was used to confirm increased Erg protein levels in Ad-Erg compared to Ad-GFP infected cells at 3 and 4 days post-transduction. GapDH was used as a loading control. (B–H) Sclerotome micromass cultures were infected with Ad-GFP (C, E, G) or Ad-Erg (D, F, H) for seven days. Alcian blue staining (C, C), phase contrast images (D, F), and Toluidine blue O staining (G, H) all show a reduction in cartilage nodule formation.

Figure 4. ERG decreases cartilage gene expression and increases *Sca1*

Quantitative real-time RT PCR using RNA isolated from sclerotome micromass cultures transduced with Ad-Erg or Ad-GFP for 3 days. No effect of Erg over-expression on *Fmod*, *Adamtsl2*, or *Scx* mRNA levels were observed. *Maf* and *Sox9* mRNA were significantly decreased and *Sca1* mRNA was significantly increased. RT-PCR data was analyzed by REST. Expression relative to Ad-GFP transduced cells is shown (set to 1.0, dashed line). HPRT was used as the normalization control. * indicates $p < 0.05$. $n = 13$ for *Maf*, *Sox9*, and *Sca1*. $n = 9$ for *Fmod* and *Scx*. $N = 8$ for *Adamtsl2*.

Figure 5. Erg acts synergistically with TGF- β to regulate specific markers for mesenchymal cell differentiation

(A, B) Quantitative real-time RT-PCR using RNA isolated from sclerotome micromass cultures transduced with Ad-Erg or Ad-GFP for two days and then left untreated or treated with 1ng/ml TGF- β for 24 hours. Expression is shown relative to untreated Ad-GFP transduced cells (set to 1.0, dashed line). TGF- β and Erg cooperated to up-regulate *Adamtsl2* and *Scx* (A) and down-regulate *c-Maf* (B). RT-PCR data analyzed by REST. HPRT was

used as the normalization control. * indicates $p < 0.05$ relative to the Ad-GFP, untreated control. The step lines indicate $p < 0.05$ between the two samples connected. $n=4$.

Figure 6. Erg and Smad3 interact in a protein complex

(A) Cells were infected with Ad-GFP or Ad-Erg virus for 48 hours. Control rabbit IgG or rabbit anti-Smad3 antibody was used for immunoprecipitation. The immuno-complexes were subjected to western blot analysis using a goat antibody to Erg. High levels of Erg were detected in Smad3 immuno-complexes from Ad-Erg infected cells relative to those from Ad-GFP infected cells or from immuno-complexes from IgG precipitated controls, which indicate some background signal likely from the IgG heavy chain. An immunoblot using a sample of the protein lysates before immunoprecipitation (input) shows overexpression of Erg in the Ad-Erg infected samples. (B) Endogenous Erg was immunoprecipitated from protein lysates using a goat anti-Erg antibody. The immuno-complexes were separated on gels for western blot analysis using a rabbit anti-Smad3 antibody. Smad3 was detected in the Erg immuno-complexes. Western blot of samples of the protein lysates before immunoprecipitation (input) show expression of Smad3.

Table 1

List of primers used for QRT-PCR.

Gene Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	product size (bp)
<i>Adamtsl2</i>	GGGCAACAATCATCTTGGTTACT	CCGTCGGTACTTGACCACT	199
<i>Erg</i>	TCACCCCTCAGTCCAAAGCTGC	TCCCCGTTGGTGCCTTCCCA	217
<i>Fmod</i>	CCTCCTGTCAACACCAACCT	GCTGCTGATGGAGAACTCATT	72
<i>Hprt1</i>	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG	142
<i>Maf</i>	GGAGACCGACCGCATCATC	TCATCCAGTAGTAGTCTTCCAGG	173
<i>Scal</i>	TGTGTTACTCAGGAGGCAGCAGTT	TAGGAGGGCAGATGGGTAAGCAA	85
<i>Scx</i>	TCTGCCTCAGCAACCAGAGAAAGT	ACTCTTCAGTGGCATCCACCTTCA	130
<i>Sox9</i>	GGCCGAAGAGGCCACGGAAC	GATTGCCCAGAGTGCTCGCCC	129