

Slug is an Essential Target of TGF β 2 Signaling in the Developing Chicken Heart

Laura A. Romano¹ and Raymond B. Runyan²

Department of Cell Biology and Anatomy, University of Arizona, Tucson, Arizona 85724

An epithelial–mesenchymal cell transformation (EMT) occurs during the development of endocardial cushions in the atrioventricular (AV) canal of the heart. This is a complex developmental process regulated by multiple extracellular signals and signal transduction pathways. It was recently shown that the transcription factor Slug is expressed in the AV canal and is required for initial steps of EMT. Treatment of AV canal explants with either antisense oligodeoxynucleotides toward Slug or anti-TGF β 2 antibody inhibited initial steps of EMT. Others have identified roles for HGF and BMP during EMT in the heart. Both HGF and BMP are known to regulate Slug in other cell types. To determine whether TGF β 2 or other signaling factors regulate Slug expression during EMT in the heart, we cultured AV canal explants in the presence of anti-TGF β 2 antibody, anti-TGF β 3 antibody, pertussis toxin, retinoic acid, noggin, or anti-HGF antibody. Only treatment with anti-TGF β 2 antibody or retinoic acid inhibited Slug expression in AV canal explants. Consistent with these data, we found that retinoic acid disrupted initial steps of EMT, while antagonists of BMP and HGF signaling disrupted later steps of EMT. Transfection of AV canal explants with Slug rescued the inhibitory effect of anti-TGF β 2 antibody but not retinoic acid on EMT. Slug is thus an essential target of TGF β 2 signaling during EMT in the developing chicken heart.

© 2000 Academic Press

Key Words: BMP; cardiac development; endocardial cushions; HGF; noggin; retinoic acid; transcription factor; valve formation; zinc finger protein.

INTRODUCTION

Early in embryonic development, the vertebrate heart is a hollow tube composed of an outer layer of myocardium and an inner layer of endocardium separated by extracellular matrix (ECM). As cardiac morphogenesis proceeds, endocardial cushions develop within the atrioventricular (AV) canal. The endocardial cushions initially consist only of ECM (Krug *et al.*, 1987). Later, in response to an inductive signal emitted by AV canal myocardium (Runyan and Markwald, 1983; Krug *et al.*, 1985, 1987), a portion of the endothelial cells lining the endocardial cushions undergo an epithelial–mesenchymal cell transformation (EMT) and migrate into the underlying ECM (Kinsella and Fitzharris, 1980). Endocardial cushion tissue eventually contributes to the leaflets of the mitral and tricuspid valves, as well as to the interatrial and interventricular septa (Markwald *et al.*, 1984; Chin *et al.*, 1992). Therefore, the study of EMT is critical to understanding how certain heart defects arise in humans.

The zinc finger protein Slug is a member of the Snail family of related transcription factors (Nieto *et al.*, 1994).

Slug is expressed by AV canal endothelial cells, as well as by mesenchymal cells within the endocardial cushions. *In vitro* experiments demonstrated that Slug is required for AV canal endothelial cells to undergo initial steps of EMT. Treatment of chicken AV canal explants with antisense Slug oligodeoxynucleotides inhibited mesenchymal cell formation. Specifically, antisense treatment prevented endothelial cell–cell separation, suggesting that Slug acts early in the EMT pathway (Romano and Runyan, 1999).

Slug also is required for the EMTs that occur during gastrulation and neural crest differentiation in chicken embryos. Epiblast cells flanking the primitive streak express Slug as they transform into mesenchymal cells and migrate down into the blastocoel during gastrulation. Presumptive neural crest cells express Slug as they transform into mesenchymal cells and migrate from the dorsal region of the neural tube. When stage 4–9 chicken embryos were cultured *in vitro* in the presence of antisense Slug oligodeoxynucleotides, mesoderm formation was impaired. Likewise, presumptive neural crest cells failed to transform into mesenchyme, resulting in a wide range of defects (Nieto *et al.*, 1994). It has been proposed that Slug regulates the expression of genes required for the changes in cell adhesion and cytoskeletal organization necessary for epithelial cells to transform into migratory mesenchymal cells. Slug also

¹ Present address: Department of Zoology, Duke University, Durham, NC 27708.

² To whom correspondence should be addressed.

may play a role in maintaining the mesenchymal cell phenotype in tissues such as the limb bud (Buxton *et al.*, 1997; Ros *et al.*, 1997).

Several signaling factors are good candidates for regulating Slug expression in the heart. For instance, TGF β 2, a member of a large superfamily of related growth factors, is expressed in both AV canal myocardium and endocardium (Potts *et al.*, 1992; Boyer *et al.*, 1999a). Treatment of AV canal explants with anti-TGF β 2 antibody inhibited initial steps of EMT including endothelial cell-cell separation (Boyer *et al.*, 1999a). G $_i$ proteins regulate intracellular signaling events by inhibiting adenylate cyclase in response to activation of a G-protein-linked receptor. Treatment of AV canal explants with pertussis toxin, an antagonist of G $_i$ protein signaling, also inhibited initial steps of EMT (Runyan *et al.*, 1990; Boyer *et al.*, 1999a). In contrast, treatment of AV canal explants with anti-TGF β 3 antibody inhibited later steps of EMT including mesenchymal cell migration (Boyer *et al.*, 1999a).

Retinoic acid also is a candidate for regulating Slug expression in the heart, although an endogenous source of retinoic acid is unknown. Mice exposed to inappropriate levels of retinoic acid, or which have a null mutation for the retinoid X receptor-alpha (RXR α), exhibit AV septal defects (Gruber *et al.*, 1996; reviewed by Smith *et al.*, 1998). BMPs and HGF have been shown to regulate AV canal development and therefore, may regulate Slug expression as well. It appears that BMP2 and TGF β 3 act synergistically to mediate certain steps of EMT in the AV canal (Yamagishi *et al.*, 1999; Nakajima *et al.*, 2000). HGF appears to regulate mesenchymal cell migration (Song *et al.*, 1999). It is especially important to consider BMPs and HGF as candidates for regulating Slug expression in the heart since these growth factors have been shown to regulate Slug expression in other tissues (Liem *et al.*, 1995; Savagner *et al.*, 1997; Selleck *et al.*, 1998).

To identify specific signaling factors that regulate Slug expression during EMT in the heart, we cultured AV canal explants in the presence of anti-TGF β 2 antibody, anti-TGF β 3 antibody, pertussis toxin, retinoic acid, noggin, or anti-HGF antibody. Semiquantitative RT-PCR and immunostaining demonstrated that only treatment with anti-TGF β 2 antibody or retinoic acid inhibited Slug expression in AV canal explants. Consistent with these data, we found that treatment of AV canal explants with retinoic acid inhibited initial steps of EMT in a dose-dependent manner. Treatment of explants with noggin (an antagonist of BMP signaling) or anti-HGF antibody inhibited later steps of EMT. Addition of exogenous BMP-2 or HGF rescued the inhibitory effects of noggin or anti-HGF antibody, respectively, on transformation.

Transfection of AV canal explants with Slug rescued the inhibitory effect of anti-TGF β 2 antibody but not retinoic acid on EMT. Therefore, Slug is an essential target of TGF β 2 signaling during EMT in the developing chicken heart. We speculate that TGF β 2 regulates Slug expression via activation of the Type III TGF β receptor, which is

required for initial steps of EMT (Brown *et al.*, 1999). We also suggest that Slug was not sufficient to overcome the inhibitory effect of retinoic acid on EMT because it is only one of several genes in the heart disrupted by retinoic acid exposure.

MATERIALS AND METHODS

Treatment of AV Canal Explants with Signaling Factors or Signaling Factor Antagonists

Collagen gels were prepared according to Potts and Runyan (1989): 1 ml of rat tail collagen (approximately 3.5 mg/ml) diluted 1:1 in water was mixed with 200 μ l of 2.2% sodium bicarbonate (Sigma, St. Louis, MO) diluted 1:1 in 10 \times Medium 199 (Gibco BRL, Gaithersburg, MD). Two hundred eighty microliters of the collagen solution was poured into each well of a Nunclon MultiDish culture dish (Nunc, Roskilde, Denmark) and allowed to solidify for at least 15 min at room temperature. Five hundred microliters of medium was applied to the collagen gels for at least 1 h at 37°C in a tissue culture incubator prior to use.

Fertilized White Leghorn chicken eggs (Rosemary's Farm, Santa Monica, CA) were incubated at 37°C to obtain embryos, which were staged according to Hamburger and Hamilton (1951). AV canals were dissected from embryonic chicken hearts in Tyrode's solution (Sigma) and cut open to expose the inner endocardial surface. The explants were placed on collagen gels so that the endocardium was in contact with the surface of the gel to facilitate endothelial cell outgrowth. After approximately 6 h of incubation at 37°C in a tissue culture incubator, the following signaling factors or signaling factor antagonists were applied to the explants: polyclonal anti-chicken TGF β 2 antibody (R&D Systems, Minneapolis, MN) (10 μ g/ml) diluted in 300 μ l of Medium 199, 300 μ l of monoclonal anti-chicken TGF β 3 antibody supernatant (Developmental Studies Hybridoma Bank, University of Iowa), pertussis toxin (Sigma) (10 ng/ml) diluted in 300 μ l of Medium 199, retinoic acid (Sigma) (10 ng/ml) diluted in 300 μ l of Medium 199, 300 μ l of noggin-containing or control conditioned medium diluted 1:1 in Medium 199, or a polyclonal anti-human HGF antibody (R&D Systems) (10 μ g/ml). Conditioned medium was prepared using parental Chinese hamster ovary (CHO) cells or CHO cells stably expressing *Xenopus* noggin (Smith and Harland, 1992). Dr. Richard Harland (University of California at Berkeley) kindly provided CHO cells. A range of retinoic acid concentrations was tested (0.5–300 ng/ml) (data not shown). Retinoic acid at a concentration of 10 ng/ml proved to most effectively inhibit EMT without any visible signs of toxicity.

After 24 h of incubation, the number of mesenchymal cells within the collagen gel was counted for each explant under an Olympus inverted microscope. Observation under the microscope revealed that both control and treated explants were viable and that the myocardium was beating. A *t* test, two-sample assuming unequal variances, was performed to compare control and treated groups of explants (Microsoft Excel software).

Semiquantitative RT-PCR

After 24 h of incubation, total RNA was extracted from selected AV canal explants using RNA Stat-60 (Tel-Test "B", Inc., Friendswood, TX) and treated with DNase (Gibco BRL). RT-PCR was then performed. The total volume for each reverse transcription reaction was 30 μ l for 2 μ g of RNA. The reaction also included 1.5 μ l of random primers (Gibco BRL), 1 μ l of 10 mM dNTPs (Gibco BRL), 20

U of rRNasin RNase inhibitor (Promega, Madison, WI), and 400 U of M-MLV reverse transcriptase in 5 \times First Strand Buffer supplied with the enzyme (Gibco BRL). cDNA was synthesized in a PTC-100 programmable thermal controller (MJ Research, Inc., Watertown, MA) under the following conditions: a 1.5-h incubation at 37°C and a 10-min incubation at 75°C.

Specific primers were designed to amplify a 358-bp Slug sequence by PCR. The primer sequences were 5' CTGCCTTCAAAATGCCAC (bp 1–14) and 3' TTGGACTGGATTCTCTCT (bp 341–358). The total volume for each PCR was 50 μ l. The reaction included 0.1 μ g of each specific primer (Integrated DNA Technologies, Inc., Coralville, IA), 0.1 μ g of cDNA, 1 μ l of 10 mM dNTPs (Gibco), 3 μ l of 25 mM magnesium chloride (Fisher Scientific, Pittsburgh, PA), and 2.5 U of *Taq* DNA Polymerase in 10 \times Assay Buffer B supplied with the enzyme (Fisher Scientific). PCR was performed in a PTC-100 programmable thermal controller under the following conditions: 30 s denaturation at 92°C, 1 min annealing at 58°C, and 1.5 min extension at 72°C. As a control for loading, RT-PCR also was performed using specific primers designed to amplify an 800-bp glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequence.

The PCR products were fractionated on a 1% agarose gel and visualized using ethidium bromide. Band densities were quantitated using NIH Image analysis software. The background density of the gel was subtracted from the peak density for each band. The area of each band measured was then multiplied by the adjusted band density to determine relative amplification levels. Twenty-seven cycles was determined to be within the linear range of both *Slug* and *GAPDH* amplification by removing and analyzing aliquots from a series of cycles (20–35). RT-PCR was then repeated for 27 cycles using RNA collected from both control and treated explants. *Slug* and *GAPDH* band densities were quantitated using NIH Image analysis software as described above. *Slug* band densities were normalized to *GAPDH* levels in the same RNA samples. The RT-PCR experiment was performed four times and average *Slug* band densities were obtained. The average *Slug* band densities obtained from treated AV canal explants were plotted relative to the average *Slug* band density obtained from control AV canal explants, which was arbitrarily set at 1.

Immunostaining of AV Canal Explants

After 24 h of incubation, selected AV canal explants were rinsed with Tyrode's solution for 30 min at room temperature and fixed with 2% paraformaldehyde overnight at 4°C. After fixation, the explants were rinsed with 1 \times PBS for 1 h at room temperature. To block nonspecific binding sites, 1 \times PBS containing 1% bovine serum albumin (Sigma) and 2% donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was applied to the explants for 30 min at 37°C. Blocking solution containing 0.2% Tween (Sigma) was then applied to the explants overnight at 4°C. A monoclonal anti-chicken Slug antibody (Developmental Studies Hybridoma Bank) was diluted 1:100 in 1 \times PBS containing 0.5% bovine serum albumin, 1% donkey serum, and 0.2% Tween and applied to the explants for 2 h at 4°C. Alternatively, mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) (1 μ g/ml) diluted in 1 \times PBS containing 0.5% bovine serum albumin, 1% donkey serum, and 0.2% Tween was applied to some of the explants as a negative control. After incubation with the primary antibody, the explants were rinsed with 1 \times PBS containing 0.2% Tween for 2 h at room temperature. AffiniPure rabbit anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc.) (10 μ g/ml) was diluted in 1 \times PBS containing 0.2% Tween and applied to the explants for 30 min

at 4°C. After incubation with the secondary antibody, the explants were rinsed as previously described. Finally, Cy5-conjugated AffiniPure goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc.) was diluted 1:300 in 1 \times PBS containing 0.2% Tween and applied to the explants for 45 min at 4°C. After incubation with the tertiary antibody, the explants were rinsed with 1 \times PBS containing 0.2% Tween for 2 h at room temperature and then with 1 \times PBS for 30 min at room temperature. After the explants were rinsed with water, the collagen gels were transferred to glass slides. Coverslips were mounted using glycerol gelatin and sealed with nail polish. The slides were stored in the dark at 4°C until viewed under a Leica confocal microscope.

Transfection of AV Canal Explants with Slug cDNA

Full-length chicken Slug cDNA (provided by Dr. Thomas Jessell at Columbia University, New York) was cloned into pIRES2-EGFP vector (Clontech, Palo Alto, CA) using *EcoRI* and *BamHI* restriction enzymes (Gibco BRL). A mixture of plasmid DNA and Lipofectamine reagent (Gibco BRL) was prepared as follows: 12 μ g of plasmid DNA (pIRES2-EGFP or Slug-pIRES2-EGFP) was diluted in 100 μ l of Medium 199 (Gibco BRL). Twelve microliters of Lipofectamine reagent was also diluted in 100 μ l of Medium 199. The plasmid DNA and Lipofectamine reagent solutions were mixed and incubated for 15 min at room temperature. An additional 800 μ l of Medium 199 was then added to the mixture.

Collagen gels were prepared as described above. AV canals were dissected from stage 14 embryonic chicken hearts in Tyrode's solution (Sigma) and cut open to expose the inner endocardial surface. The explants were incubated in the mixture of plasmid DNA and Lipofectamine reagent for 30 min at 37°C in a tissue culture incubator. After 30 min of incubation, the explants were placed on collagen gels and returned to the tissue culture incubator. After approximately 6, 18, and 30 h of incubation, a polyclonal anti-chicken TGF β 2 antibody (R&D Systems) (10 μ g/ml) or retinoic acid (Sigma) (10 ng/ml) diluted in 300 μ l of Medium 199 was applied to the explants. After 48 h of incubation, the number of mesenchymal cells within the collagen gel was counted for each explant under an Olympus inverted microscope. Observation under the microscope revealed that transfected explants were viable and that the myocardium was beating. A *t* test, two-sample assuming unequal variances, was performed to compare control and treated groups of explants (Microsoft Excel software).

RESULTS

Retinoic Acid Inhibits Initial Steps of Epithelial-Mesenchymal Cell Transformation

The transcription factor Slug is expressed in the AV canal and is required for initial steps of EMT. Slug expression within AV canal endocardium appears to be an early response to a signal emitted by AV canal myocardium (Romano and Runyan, 1999). Previous studies have implicated several signaling factors including TGF β 2, TGF β 3, BMP, and HGF as components of the inductive signal (Potts *et al.*, 1992; Boyer *et al.*, 1999a; Song *et al.*, 1999; Yamagishi *et al.*, 1999). TGF β 2 is required along with Slug for initial steps of EMT, whereas TGF β 3 is required for later steps of EMT (Boyer *et al.*, 1999a). In this study, we performed experiments to determine if BMP and HGF are required for

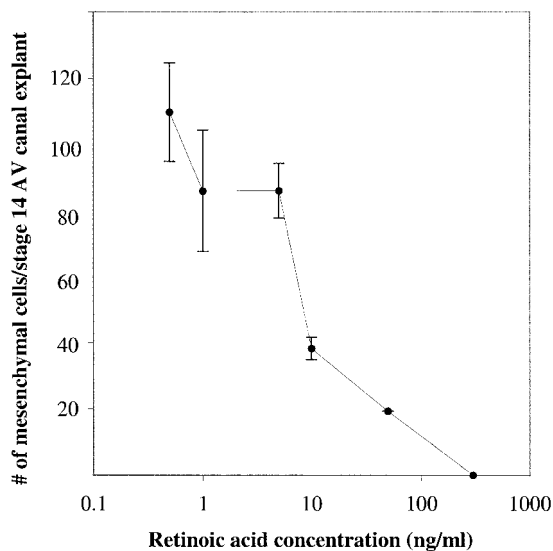


FIG. 1. Retinoic acid inhibited initial steps of epithelial-mesenchymal cell transformation. Stage 14 AV canal explants were cultured on collagen gels in the presence of retinoic acid. After 24 h of incubation, the number of mesenchymal cells within the collagen gel was counted for each explant. Treatment of explants with retinoic acid resulted in a statistically significant ($P < 0.05$) decrease in mesenchymal cell formation compared to untreated explants. Error bars indicate the standard errors ($N > 10$).

specific steps of EMT. In addition, we tested the effect of retinoic acid on EMT. Signaling factors required for initial steps of EMT were hypothesized to be good candidates for regulating Slug expression in the heart.

AV canals were dissected from Hamburger and Hamilton (1951) stage 14 chicken embryos and cultured on collagen gels in medium containing retinoic acid. After 24 h of incubation, the number of mesenchymal cells beneath the surface of the collagen gel was counted for each explant. Treatment of explants with retinoic acid resulted in a statistically significant decrease in mesenchymal cell formation compared to untreated explants (Fig. 1). Specifically, retinoic acid treatment resulted in approximately 63% fewer mesenchymal cells than untreated explants.

Retinoic acid interfered with initial steps of EMT, including endothelial cell-cell separation. That is, the AV canal endothelial cells of retinoic acid-treated explants (Fig. 2I) were smaller and remained in tight contact with one another compared to the AV canal endothelial cells of control explants (Fig. 2A). In addition, fewer mesenchymal cells were observed within the collagen gel (Fig. 2J).

We attempted to rescue the inhibitory effect of retinoic acid on EMT by adding exogenous TGF β 2. AV canals were dissected from stage 14 chicken embryos and cultured on collagen gels in medium containing both retinoic acid and TGF β 2. After 24 h of incubation, the number of mesenchymal cells within the collagen gel was counted for each explant. Treatment of explants with a combination of

retinoic acid and TGF β 2 produced no change in mesenchymal cell formation compared to explants treated only with retinoic acid (Fig. 3). Therefore, TGF β 2 was not sufficient to overcome the inhibitory effect of retinoic acid on EMT.

Noggin, an Antagonist of BMP Signaling, Inhibits Later Steps of Epithelial-Mesenchymal Cell Transformation

Previous work suggests that BMP signaling is required for EMT in the heart (Yamagishi *et al.*, 1999). Therefore, we tested the effect of noggin, an antagonist of BMP signaling, on EMT. AV canals were dissected from stage 13, 14, 15, or 16 chicken embryos and cultured on collagen gels in noggin-containing or control conditioned medium. After 24 h of incubation, the number of mesenchymal cells within the collagen gel was counted for each explant. Noggin had no significant effect on mesenchymal cell formation in stage 13, 14, or 15 explants. However, treatment of stage 16 explants with noggin resulted in a statistically significant decrease in mesenchymal cell formation compared to untreated explants or explants cultured in control conditioned medium (Fig. 4). Specifically, treatment of stage 16 explants with noggin resulted in approximately 30% fewer mesenchymal cells than explants cultured in control conditioned medium. The inhibitory effect of noggin on stage 16 explants was rescued by the addition of exogenous BMP-2 (Fig. 4).

The AV canal endothelial cells of both control (Figs. 2A and 2E) and noggin-treated (Fig. 2C) stage 16 explants formed a large monolayer on the surface of the collagen gel and eventually separated from one another. However, significantly fewer mesenchymal cells were observed within the collagen gel in the presence of noggin (Fig. 2D) compared to control explants (Figs. 2B and 2F). Therefore, BMP signaling appears to be required only for later steps of EMT such as mesenchymal cell migration. This is supported by the finding that noggin had no discernible effect on stage 13, 14, or 15 explants, although AV canal endothelial cells begin to receive an inductive signal from AV canal myocardium as early as stage 13+ (Ramsdell and Markwald, 1998).

Anti-HGF Antibody Inhibits Later Steps of Epithelial-Mesenchymal Cell Transformation

HGF signaling also may be required for AV canal endothelial cells to undergo EMT (Song *et al.*, 1999). AV canals were dissected from Hamburger and Hamilton (1951) stage 14, 15, 16, or 17 chicken embryos and cultured on collagen gels in medium containing anti-HGF antibody. After 24 h of incubation, the number of mesenchymal cells within the collagen gel was counted for each explant. The anti-HGF antibody had no effect on mesenchymal cell formation in stage 14, 15, or 16 explants. However, treatment of stage 17 explants with anti-HGF antibody resulted in a statistically significant decrease in mesenchymal cell formation compared to untreated explants (Fig. 5). Specifically, treatment of stage 17 explants with anti-HGF antibody resulted in approximately 28% fewer mes-

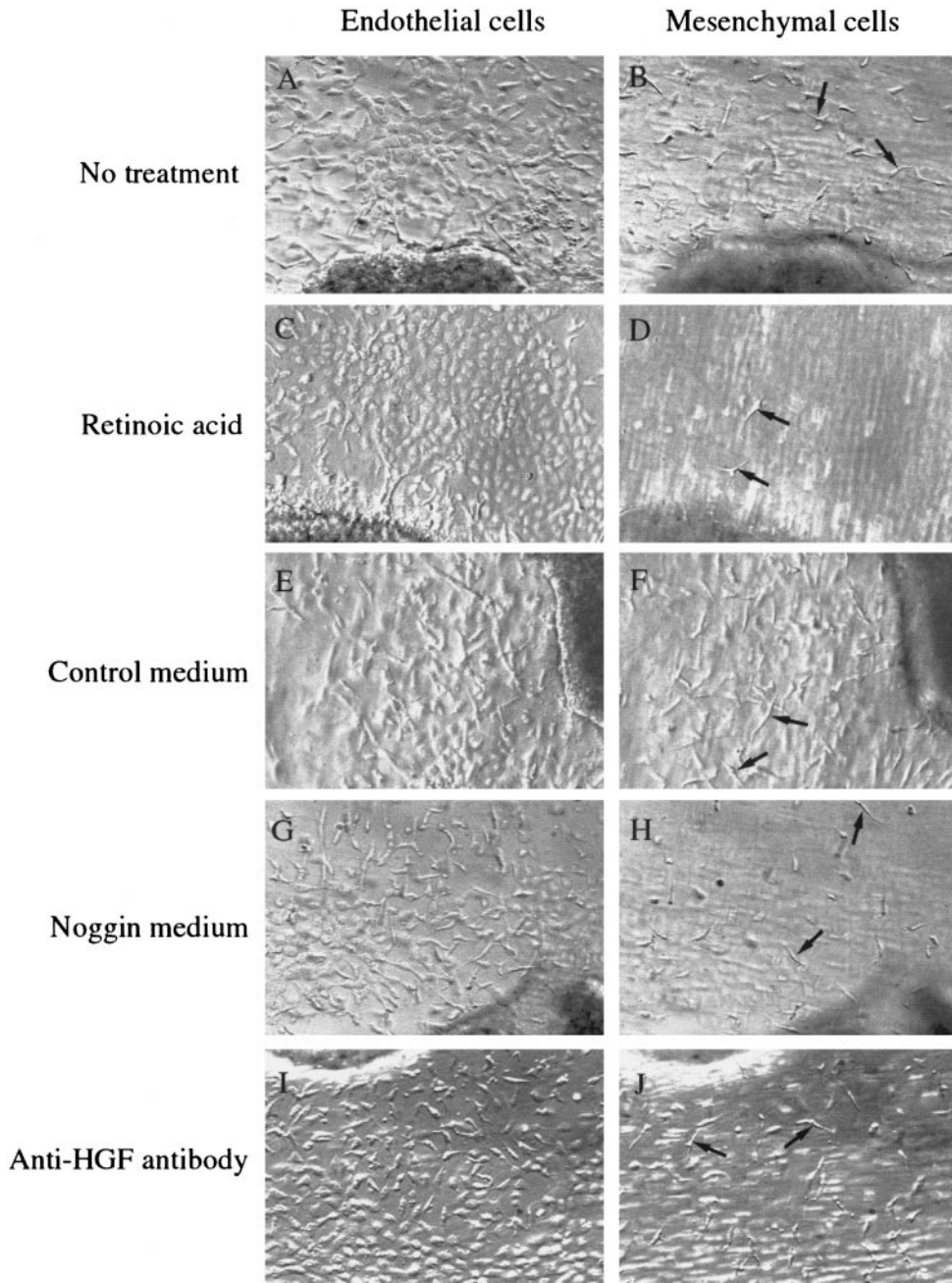


FIG. 2. Retinoic acid inhibited initial steps of epithelial-mesenchymal cell transformation including endothelial cell-cell separation, while noggin and anti-HGF antibody inhibited later steps of epithelial-mesenchymal cell transformation including mesenchymal cell migration (endothelial cells, A, C, E, G, I; mesenchymal cells, B, D, F, H, J). The AV canal endothelial cells of untreated explants (A), explants cultured in noggin-containing (G) or control (E) conditioned medium, or explants treated with anti-HGF antibody (I) exhibited endothelial cell-cell separation. In contrast, the AV canal endothelial cells of explants treated with retinoic acid (C), fewer mesenchymal cells were observed beneath the surface of the collagen gel when explants were treated with retinoic acid (D), noggin (H), or anti-HGF antibody (J), compared to untreated explants (B) or explants cultured in control conditioned medium (F).

enchymal cells than in untreated explants. The inhibitory effect of the anti-HGF antibody on stage 17 explants was rescued by the addition of exogenous HGF (Fig. 5).

The AV canal endothelial cells of both control explants (Fig. 2A) and explants treated with anti-HGF antibody (Fig. 2G) formed a large monolayer on the surface of the collagen

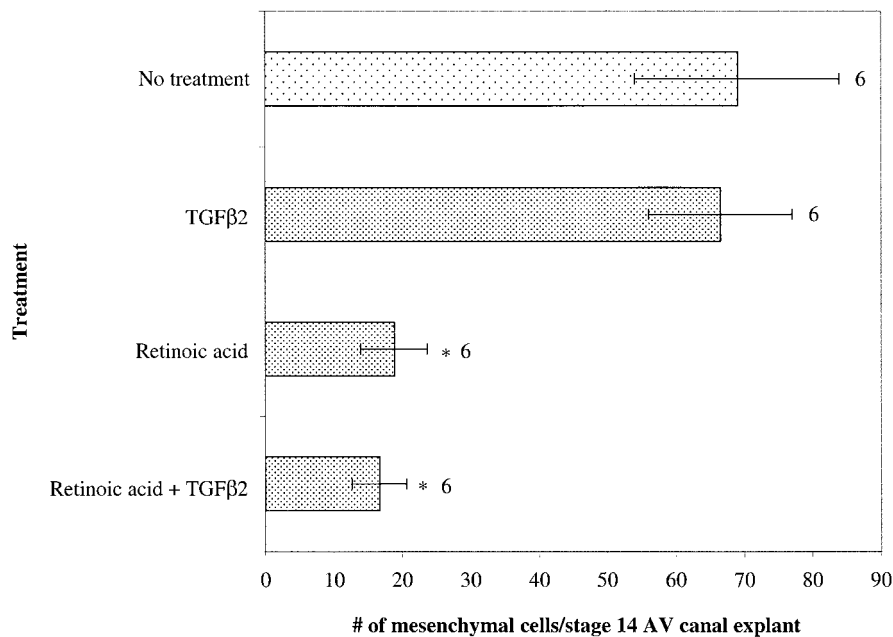


FIG. 3. TGFβ2 did not rescue the inhibitory effect of retinoic acid on epithelial–mesenchymal cell transformation. Stage 14 AV canal explants were cultured on collagen gels in the presence of retinoic acid alone or a combination of retinoic acid and TGFβ2. After 24 h of incubation, the number of mesenchymal cells within the collagen gel was counted for each explant. Treatment of explants with a combination of retinoic acid and TGFβ2 resulted in a statistically significant ($P < 0.05$) decrease in mesenchymal cell formation compared to untreated explants or explants treated with only TGFβ2. Error bars indicate the standard errors.

gel and eventually separated from one another. However, significantly fewer mesenchymal cells were observed within the collagen gel when stage 17 explants were treated with anti-HGF antibody (Fig. 2H) compared to control explants (Fig. 2B). Therefore, like BMP, HGF may be required only for later steps of EMT such as mesenchymal cell migration. This is supported by the finding that the anti-HGF antibody had no discernible effect on stage 14, 15, or 16 explants.

Anti-TGFβ2 Antibody or Retinoic Acid Inhibits Slug Expression in AV Canal Explants

To determine if TGFβ2 or possibly other signaling factors regulate Slug expression during EMT in the heart, we cultured AV canal explants on collagen gels in the presence of anti-TGFβ2 antibody, anti-TGFβ3 antibody, pertussis toxin, retinoic acid, noggin, or anti-HGF antibody. RNA was collected after 24 h of incubation and used to perform semiquantitative RT-PCR. Specifically, RT-PCR was performed using specific primers designed to amplify a 358-bp Slug sequence or an 800-bp GAPDH sequence. A single band of the expected size was detected with each set of primers. No bands were detected when RT-PCR was performed without reverse transcriptase (data not shown). Twenty-seven cycles was determined to be within the linear range of both Slug and GAPDH amplification by removing and analyzing aliquots from a series of cycles

(20–35). RT-PCR was then repeated for 27 cycles using RNA collected from both untreated and treated explants. Quantification of Slug band density normalized to GAPDH levels from the same RNA samples demonstrated that only treatment with anti-TGFβ2 antibody or retinoic acid resulted in a statistically significant decrease in Slug mRNA expression compared to untreated explants (Fig. 6).

We also performed immunostaining to show that treatment with anti-TGFβ2 antibody or retinoic acid resulted in a decrease in Slug protein expression. After 24 h of incubation, the explants were stained with a monoclonal anti-chicken Slug antibody. The AV canal endothelial cells of untreated explants express Slug, as do mesenchymal cells within the collagen gel (Fig. 7B). However, staining was reduced within the endothelium of explants treated with anti-TGFβ2 antibody or retinoic acid (Figs. 7B and 7C). No staining was observed when explants were stained for mouse IgG as a negative control (Fig. 7A).

Slug Rescues the Inhibitory Effect of Anti-TGFβ2 Antibody but Not Retinoic Acid on Epithelial–Mesenchymal Cell Transformation

Treatment of AV canal explants with anti-TGFβ2 antibody or retinoic acid inhibited Slug expression, suggesting that Slug is either directly or indirectly regulated by these signaling factors. We proceeded to test whether Slug is sufficient to overcome the inhibitory effect of anti-TGFβ2

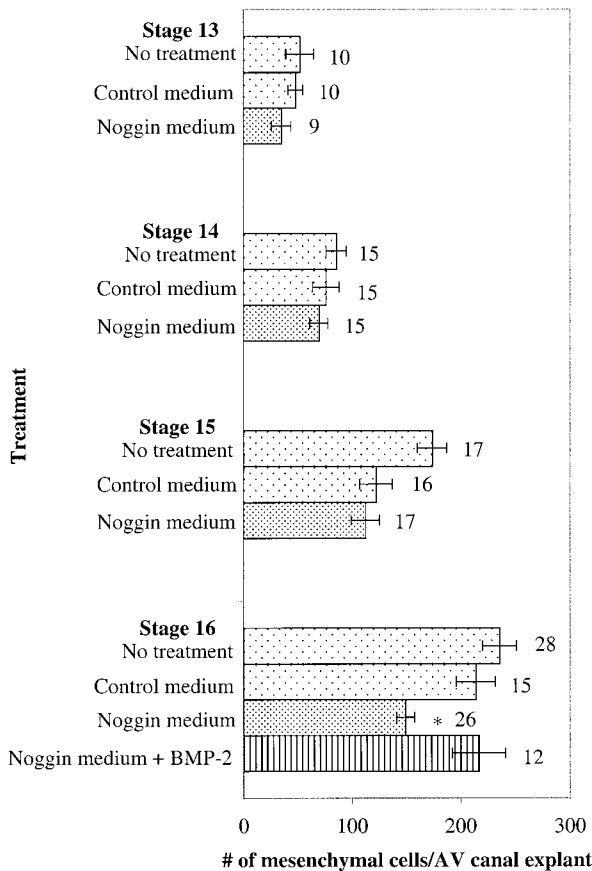


FIG. 4. Noggin, an antagonist of BMP signaling, inhibited later steps of epithelial-mesenchymal cell transformation. Stage 13, 14, 15, or 16 AV canal explants were cultured on collagen gels in noggin-containing or control conditioned medium. After 24 h of incubation, the number of mesenchymal cells within the collagen gel was counted for each explant. Noggin had no significant effect on mesenchymal cell formation in stage 13, 14, or 15 explants. However, treatment of stage 16 AV canal explants with noggin resulted in a statistically significant ($P < 0.05$) decrease in mesenchymal cell formation compared to untreated explants or explants cultured in control conditioned medium. The inhibitory effect of noggin on stage 16 explants was rescued by the addition of exogenous BMP-2. Error bars indicate the standard errors.

antibody or retinoic acid on initial steps of EMT. AV canals were dissected from stage 14 embryonic chicken hearts and transfected with a plasmid encoding full-length Slug and GFP (green fluorescent protein) (Fig. 8). The plasmid was preincubated with Lipofectamine to facilitate delivery into the cells. The transfected tissue was then cultured on collagen gels in the presence of anti-TGFβ2 antibody or retinoic acid. After 48 h of incubation, the number of mesenchymal cells within the collagen gel was counted for each explant.

Slug partially rescued the inhibitory effect of anti-TGFβ2 antibody but not retinoic acid on EMT (Fig. 9). In the

presence of anti-TGFβ2 antibody, transfection of explants with Slug resulted in approximately 28% more mesenchymal cells than explants transfected with control plasmid. In contrast, in the presence of retinoic acid there was no significant difference between explants transfected with Slug and explants transfected with control plasmid. Therefore, Slug is sufficient to overcome the inhibitory effect of anti-TGFβ2 antibody but not retinoic acid on initial steps of EMT.

DISCUSSION

The transcription factor Slug is required for initial steps of the EMT which occur during endocardial cushion formation (Romano and Runyan, 1999). In the present study, we sought to identify signaling factors that regulate Slug expression in the heart in an effort to establish a pathway of genes required for EMT.

Several signaling factors are required for initial steps of EMT and, therefore, could potentially regulate Slug expression in the heart. For instance, TGFβ2 and G_i proteins are required for AV canal endothelial cells to undergo initial steps of EMT. Treatment of AV canal explants with anti-TGFβ2 antibody or pertussis toxin, an antagonist of G_i protein signaling, inhibited initial steps of EMT including endothelial cell-cell separation (Boyer *et al.*, 1999a; Runyan *et al.*, 1990). Retinoic acid also was a good candidate for regulating Slug expression in the heart. Retinoic acid, the metabolically active form of vitamin A, alters gene expression by binding to retinoic acid receptors and retinoid X receptors (RXRs), members of the steroid superfamily of ligand-activated transcription factors (reviewed by Chambon, 1996). RXRα^{-/-} mouse embryos exhibit AV septal defects (Gruber *et al.*, 1996). Likewise, AV septal defects arise when embryos are exposed to inappropriate levels of retinoic acid (reviewed by Smith *et al.*, 1998). In this study, we found that treatment of AV canal explants with retinoic acid inhibited initial steps of EMT, including endothelial cell-cell separation, consistent with the fact that fewer mesenchymal cells were found within the endocardial cushions of chicken embryos exposed to retinoic acid (Bouman *et al.*, 1998). Recent findings suggest that retinoic acid inhibits EMT by interfering with the inductive signal provided by AV canal myocardium (Yan *et al.*, 2000). Retinoic acid also may directly affect the expression of genes in the AV canal endothelium.

Additional signaling factors are required for EMT in the heart. For instance, TGFβ3 is required for later steps of EMT including mesenchymal cell transformation (Boyer *et al.*, 1999a). BMP and HGF signaling also are required for EMT. BMPs are members of the TGFβ superfamily of related growth factors. BMP-2 is expressed in the AV canal myocardium of chicken embryos (Yamagishi *et al.*, 1999). Treatment of AV canal explants with an antisense BMP-2 oligodeoxynucleotide inhibited EMT (Yamagishi *et al.*, 1999). Recently, Nakajima *et al.* (2000) demonstrated that a combination of TGFβ3 and BMP2 stimulated competent

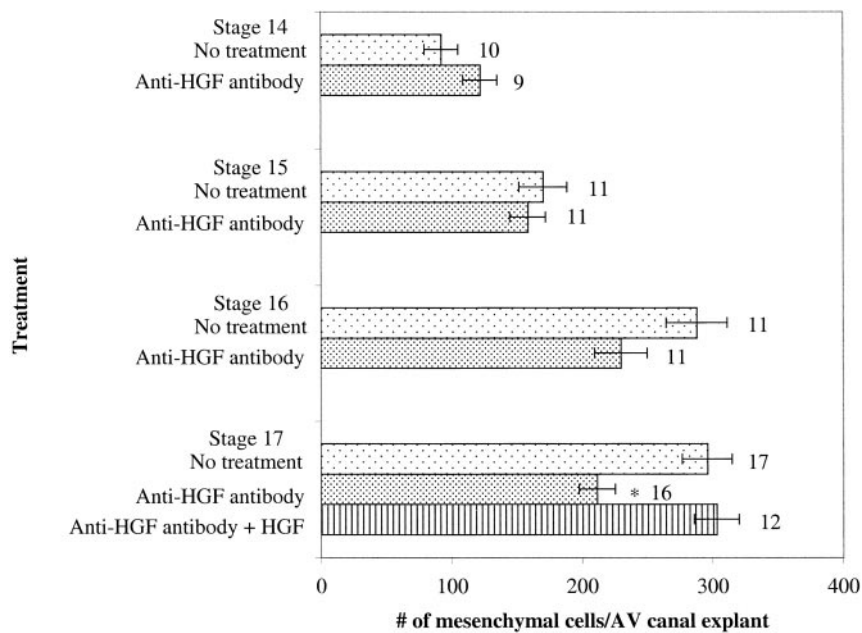


FIG. 5. Anti-HGF antibody inhibited later steps of epithelial-mesenchymal cell transformation. Stage 14, 15, 16, or 17 AV canal explants were cultured on collagen gels in the presence of anti-HGF antibody. After 24 h of incubation, the number of mesenchymal cells within the collagen gel was counted for each explant. Anti-HGF antibody had no significant effect on mesenchymal cell formation in stage 14, 15, or 16 explants. However, treatment of stage 17 AV canal explants with anti-HGF antibody resulted in a statistically significant ($P < 0.05$) decrease in mesenchymal cell formation compared to untreated explants. The inhibitory effect of anti-HGF antibody on stage 17 explants was rescued by the addition of exogenous HGF. Error bars indicate the standard errors.

endothelial cells to undergo EMT. As pharmacological doses of any TGF β isoform can mediate EMT (Potts and Runyan, 1989; Nakajima *et al.*, 1998), the significance of a particular TGF β isoform in this combination is limited. However, these data show a synergy between one or more TGF β s and BMP. In the present study, we found that treatment of AV canal explants with noggin, an antagonist of BMP signaling, inhibited only later steps of EMT, including mesenchymal cell migration. The growth factor HGF is expressed in AV canal myocardium while its receptor, c-Met, is expressed in AV canal endothelium. AV canal endothelial cells cultured *in vitro* in the absence of AV canal myocardium undergo EMT in response to exogenous HGF (Song *et al.*, 1999). This is accompanied by an increase in the expression of urokinase, a protease implicated in cell motility and migration (McGuire and Alexander, 1993). We found that treatment of AV canal explants with anti-HGF antibody also inhibited later steps of EMT.

Treatment of AV canal explants with anti-TGF β 3 antibody, noggin, or anti-HGF antibody did not inhibit Slug expression, consistent with the finding that these signaling factors are required only for later steps of EMT in the heart. However, BMP and HGF signaling have been shown to regulate Slug expression in other tissues. BMP signaling from adjacent nonneural ectoderm regulates Slug expression in the neural crest of chicken embryos (Liem *et al.*, 1995; Selleck *et al.*, 1998). Previous work demonstrated that

Slug also is a downstream target of HGF signaling during the transformation of rat NBT-II bladder carcinoma cells. The addition of exogenous HGF can induce NBT-II cells to undergo EMT *in vitro*. NBT-II cells transfected with Slug cDNA completed initial steps of transformation. However, NBT-II cells transfected with antisense Slug cDNA did not transform into mesenchymal cells and resisted induction by HGF (Savagner *et al.*, 1997). Although Slug appears to play a conserved role in EMT, these data suggest that the signaling factors that regulate Slug expression during EMT are tissue-dependent.

We found that only treatment of explants with anti-TGF β 2 antibody or retinoic acid inhibited Slug expression. These data are consistent with the fact that TGF β 2 is required along with Slug for initial steps of EMT. In addition, they are consistent with the fact that retinoic acid was shown in this study to inhibit initial steps of EMT. Retinoic acid has been shown to inhibit Slug expression in the chicken limb bud as well. Within the limb bud, Slug is expressed in the progress zone, an area of undifferentiated, rapidly proliferating mesenchymal cells subjacent to the apical ectodermal ridge (Buxton *et al.*, 1997; Ros *et al.*, 1997). Implantation of retinoic acid beads inhibited Slug expression in the progress zone (Buxton *et al.*, 1997). It is interesting to note that neural crest defects are associated with exposure of embryos to abnormal levels of retinoic acid (reviewed by Smith *et al.*, 1998). It is possible that

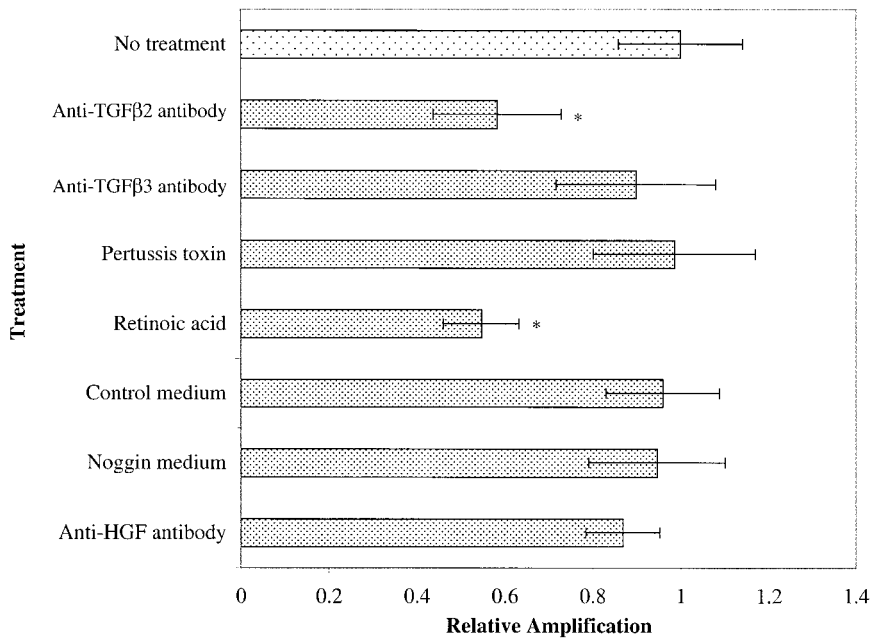


FIG. 6. Anti-TGFβ2 antibody or retinoic acid inhibited Slug mRNA expression in AV canal explants. Semiquantitative RT-PCR was performed using RNA collected from untreated explants; stage 14 explants treated with anti-TGFβ2 antibody, anti-TGFβ3 antibody, pertussis toxin, or retinoic acid; stage 16 explants cultured in noggin-containing or control conditioned medium; or stage 17 explants treated with anti-HGF antibody. Treatment of explants with anti-TGFβ2 antibody or retinoic acid resulted in a statistically significant ($P < 0.05$) decrease in Slug mRNA expression compared to untreated explants. Error bars indicate the standard errors ($N = 4$).

retinoic acid also disrupts Slug expression in presumptive neural crest cells, including those that contribute to the outflow tract region of the heart.

Transfection of AV canal explants with Slug did not rescue the inhibitory effect of retinoic acid on EMT. Slug may not have been sufficient to overcome the inhibitory effect of retinoic acid on EMT because it was only one of

several genes disrupted by retinoic acid exposure. Exposure of embryos to abnormal levels of retinoic acid appears to have a widespread effect on gene expression. For instance, abnormal levels of retinoic acid have been shown to disrupt the development of many tissues, including the neural crest, heart, eyes, limbs, and nervous system (reviewed by Smith *et al.*, 1998). The recent demonstration that retinoic

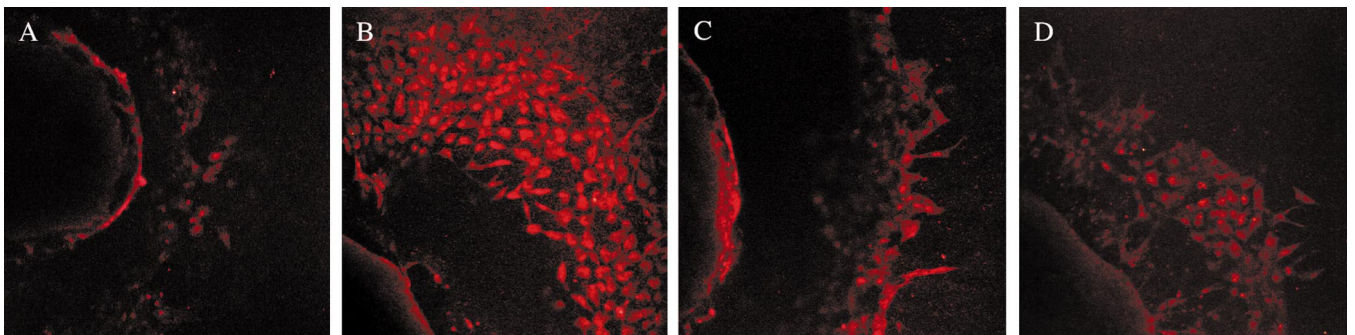


FIG. 7. Anti-TGFβ2 antibody or retinoic acid inhibited Slug protein expression in AV canal explants. (A) The AV canal endothelial cells of an untreated stage 14 AV canal explant cultured on a collagen gel for 24 h and stained for normal IgG as a negative control. (B) The AV canal endothelial cells of an untreated explant stained for Slug. The AV canal endothelial cells as well as the mesenchymal cells within the collagen gel express Slug. (C) The AV canal endothelial cells of an explant treated with anti-TGFβ2 antibody and stained for Slug. (D) The AV canal endothelial cells of an explant treated with retinoic acid and stained for Slug. Staining within the AV canal endothelium was reduced in the presence of anti-TGFβ2 antibody or retinoic acid.

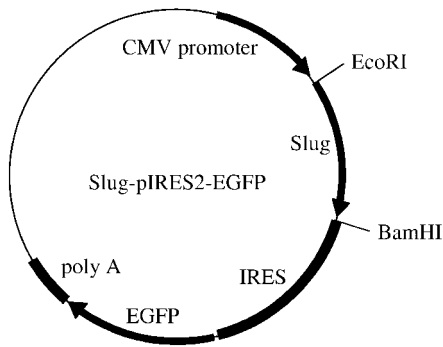


FIG. 8. A plasmid encoding full-length Slug and GFP was constructed to rescue AV canal explants treated with anti-TGF β 2 antibody or retinoic acid. Full-length Slug cDNA (829 bp) was cloned into the Clontech bicistronic expression vector pIRES2-EGFP (5.3 kb) using *EcoRI* and *BamHI* restriction enzymes. This vector permits both Slug and EGFP to be translated from a single RNA due to the presence of an internal ribosome entry site (IRES).

acid disrupts ECM secretion by the myocardium suggests that expression of Slug is unable to overcome the loss of one or more components of the inductive signal provided by the myocardium (Yan *et al.*, 2000).

In contrast, Slug is sufficient to rescue the inhibitory effect of anti-TGF β 2 antibody on EMT. Therefore, Slug appears to be an essential target of TGF β 2 signaling during initial steps of EMT in the heart. TGF β 2 signaling may regulate Slug expression through activation of the Type III TGF β receptor. Treatment of AV canal explants with a blocking antibody specific for the Type III TGF β receptor inhibited initial steps of EMT (Brown *et al.*, 1999). In contrast, treatment of AV canal explants with a blocking antibody specific for the Type II TGF β receptor inhibited distinctly later steps of EMT (Brown *et al.*, 1996). Thus, TGF β 2 appears to regulate initial steps of EMT via activation of the Type III TGF β receptor, while TGF β 3 appears to regulate later steps of EMT via activation of the Type II TGF β receptor. The Type III TGF β receptor, also known as betaglycan, has no known signaling domain (Wang *et al.*, 1991; Lopez-Casillas *et al.*, 1991) and was presumed only to "present" ligand to other TGF β receptors (Lopez-Casillas *et al.*, 1993). However, recent work suggests that the Type III TGF β receptor is capable of signal transduction (Brown *et al.*, 1999). Regulation of Slug expression by TGF β 2 but not TGF β 3 demonstrates the ability of target AV canal endothelial cells to distinguish between the different endogenous TGF β signal transduction pathways involved in EMT and is consistent with the suggestion of regulation by betaglycan. This pattern of ligand and receptor activity (i.e.,

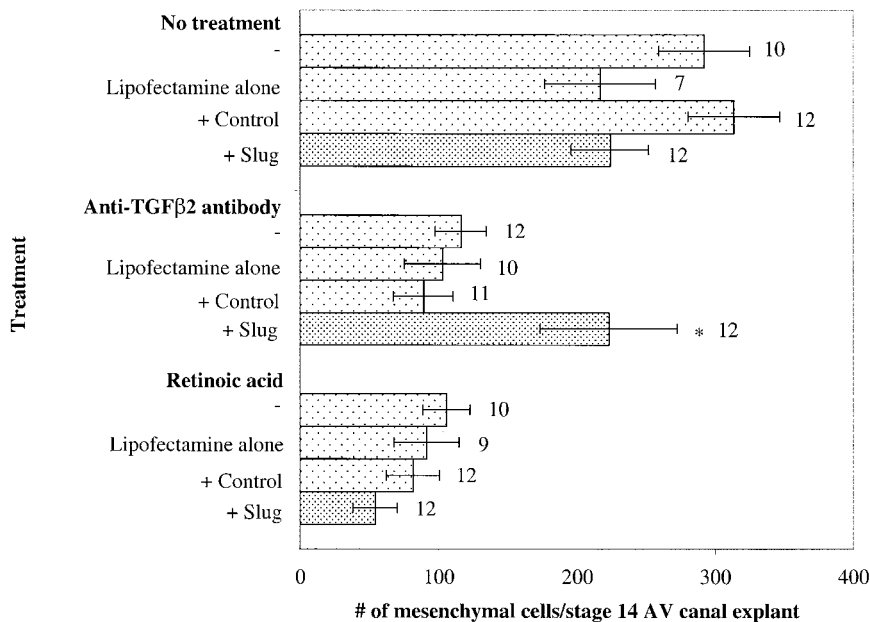


FIG. 9. Slug rescued the inhibitory effect of anti-TGF β 2 antibody but not retinoic acid on epithelial-mesenchymal cell transformation. AV canals were dissected from stage 14 chicken embryo hearts and transfected with a plasmid encoding full-length Slug and GFP. The explants were incubated for 48 h on collagen gels in the presence of anti-TGF β 2 antibody or retinoic acid. The number of mesenchymal cells within the collagen gel was then counted for each explant. Transfection of explants with Slug resulted in a statistically significant increase in mesenchymal cell formation ($P < 0.05$) in the presence of anti-TGF β 2 antibody, compared to explants transfected with control plasmid. No significant difference was observed between explants transfected with Slug and explants transfected with control plasmid in the presence of retinoic acid.

TGFβ2 with TGFβ Type III receptor and TGFβ3 with TGFβ Type II receptor) is further demonstrated by specific regulation of a variety of markers in cardiac cushion development (Boyer *et al.*, 1999b; Boyer *et al.*, in preparation).

In summary, several signaling factors are expressed in the AV canal and are required for EMT. Previous studies along with data presented here indicate that these signaling factors are required for specific steps of EMT (Boyer *et al.*, 1999a). TGFβ3, BMP, and HGF signaling is required for later steps of EMT. In contrast, TGFβ2 and G_i protein signaling is required along with Slug for initial steps of EMT. Our data indicate that AV canal endothelial cells are also sensitive to retinoic acid during initial steps of EMT. However, only TGFβ2 and retinoic acid were shown to regulate Slug expression in the heart. Although G_i protein signaling is required along with Slug for initial steps of EMT, pertussis toxin failed to inhibit Slug expression. The signaling factors involved in initial steps of EMT thus appear to regulate the expression of different or overlapping sets of genes. Additional studies are necessary to identify specific G protein-linked receptors and their downstream targets in the heart.

These data contribute to a model in which AV canal endothelial cells undergo EMT in response to multiple, independent signaling pathways. Identification of additional components of these signal transduction pathways will contribute to a better understanding of EMT. For instance, several transcription factors including Brachyury, Msx-1, and Mox-1 are expressed in the heart and have been shown to play a role during EMT in other tissues (reviewed by Huang *et al.*, 1995). One or more of these transcription factors may be required along with Slug for the EMT which occurs in the AV canal. Future studies of EMT also appear to be critical for understanding metastasis. Interestingly, Snail, a transcription factor related to Slug, was recently shown to repress the expression of E-cadherin in invasive carcinoma tissues in mouse embryos (Batlle *et al.*, 2000; Cano *et al.*, 2000).

ACKNOWLEDGMENTS

We thank Dr. Richard Harland at the University of California, Berkeley, for providing the control and noggin-expressing CHO cells. We thank Dr. Robert Baker at the University of Arizona for preparing the conditioned media. We thank Dr. Thomas Jessell at Columbia University for providing us with a full-length Slug clone. Finally, we thank Dr. Allan Sinning at the University of Mississippi for sharing information on retinoic acid prior to publication. This study was supported by the ARCS Foundation (L.A.R.) and NIH Grant HL58696 (R.B.R.).

REFERENCES

Batlle, E., Sancho, E., Franci, C., Dominguez, D., Monfar, M., Baulida, J., and de Herreros, A. G. (2000). The transcription factor Snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat. Cell Biol.* **2**, 84–89.

Bouman, H. G., Broekhuizen, M. L., Baasten, A. M., Gittenberger-de Groot, A. C., and Wenink, A. C. (1998). Diminished growth of atrioventricular cushion tissue in stage 24 retinoic acid-treated chicken embryos. *Dev. Dyn.* **213**, 50–58.

Boyer, A. S., Ayerinskas, I. I., Vincent, E. B., McKinney, L. A., Weeks, D. L., and Runyan, R. B. (1999a). TGFβ-2 and TGFβ-3 have separate and sequential activities during epithelial-mesenchymal cell transformation in the embryonic heart. *Dev. Biol.* **208**, 530–545.

Boyer, A. S., Erickson, C. P., and Runyan, R. B. (1999b). Epithelial-mesenchymal transformation in the embryonic heart is mediated through distinct pertussis toxin-sensitive and TGFβ signal transduction mechanisms. *Dev. Dyn.* **214**, 81–91.

Brown, C. B., Boyer, A. S., Runyan, R. B., and Barnett, J. V. (1996). Antibodies to the type II TGFβ receptor block cell activation and migration during atrioventricular cushion transformation in the heart. *Dev. Biol.* **174**, 248–257.

Brown, C. B., Boyer, A. S., Runyan, R. B., and Barnett, J. V. (1999). Requirement of Type III TGF-β receptor for endocardial cell transformation in the heart. *Science* **283**, 2080–2082.

Buxton, P. G., Kostakopoulou, K., Brickell, P., Thorogood, P., and Ferretti, P. (1997). Expression of the transcription factor Slug correlates with growth of the limb bud and is regulated by FGF-4 and retinoic acid. *Int. J. Dev. Biol.* **41**, 559–568.

Cano, A., Perez-Moreno, M. A., Rodrigo, I., Locascio, A., Blanco, M. J., del Barrio, M. G., Portillo, and Nieto, M. A. (2000). The transcription factor Snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat. Cell Biol.* **2**, 76–83.

Carmona, R., Gonzalez-Iriarte, M., Macias, D., Perez-Pomares, J. M., Garcia-Garrido, L., and Munoz-Chapuli, R. (2000). Immunolocalization of the transcription factor Slug in the developing heart. *Anat. Embryol.* **201**, 103–109.

Chambon, P. (1996). A decade of molecular biology of retinoic acid receptors. *FASEB J.* **10**, 940–954.

Chin, C., Gandour-Edwards, R., Oltjen, S., and Choy, M. (1992). Fate of the atrioventricular endocardial cushions in the developing chick heart. *Pediatr. Res.* **32**, 390–393.

Gruber, P. J., Kubalak, S. W., Pexieder, T., Sucov, H. M., Evans, R. M., and Chien, K. R. (1996). RXRα deficiency confers genetic susceptibility for aortic sac, conotruncal, atrioventricular cushion, and ventricular muscle defects in mice. *J. Clin. Invest.* **98**, 1332–1343.

Hamburger, V., and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49–92.

Huang, J.-X., Potts, J. D., Vincent, E. B., Weeks, D. L., and Runyan, R. B. (1995). Mechanisms of cell transformation in the embryonic heart. *Ann. N. Y. Acad. Sci.* **752**, 317–330.

Krug, E. L., Runyan, R. B., and Markwald, R. R. (1985). Protein extracts from early embryonic hearts initiate cardiac endothelial cytodifferentiation. *Dev. Biol.* **112**, 414–426.

Krug, E. L., Mjaatvedt, C. H., and Markwald, R. R. (1987). Induction of embryonic cardiac endothelial differentiation into mesenchyme by myocardially-derived extracellular proteins. *Dev. Biol.* **120**, 348–355.

Liem, K. F., Tremmi, G., Roelink, H., and Jessell, T. M. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* **82**, 969–979.

Lopez-Casillas, F., Cheifetz, S., Doody, J., Andres, J. L., Lane, W. S., and Massague, J. (1991). Structure and expression of the membrane proteoglycan betaglycan, a component of the TGFβ receptor system. *Cell* **67**, 785–795.

- Lopez-Casillas, F., Wrana, J. L., and Massague, J. (1993). Betaglycan presents ligand to the TGF β signaling receptor. *Cell* **73**, 1435–1444.
- Markwald, R. R., Kitten, G. T., Runyan, R. B., Funderburg, F. M., Bernanke, D. H., and Brauer, P. R. (1984). Use of three-dimensional collagen gel culture to study cell:matrix interactions in heart development. In "Role of the Extracellular Matrix in Development" (R. Trelstad, Ed.), Vol. 42, pp. 323–350. Alan R. Liss, Inc., New York.
- McGuire, P. G., and Alexander, S. M. (1993). Inhibition of urokinase synthesis and cell-surface binding alters the migratory behavior of embryonic endocardial-derived mesenchymal cells in vitro. *Development* **118**, 931–939.
- Nakajima, Y., Yamagishi, T., Nakamura, H., Markwald, R. R., and Krug, E. L. (1998). An autocrine function for transforming growth factor (TGF)- β 3 in the transformation of atrioventricular canal endocardium into mesenchyme during chick heart development. *Dev. Biol.* **194**, 99–113.
- Nakajima, Y., Yamagishi, T., Hokari, S., and Nakamura, H. (2000). Mechanisms involved in vavuloseptal endocardial cushion formation in early cardiogenesis: Roles of transforming growth factor (TGF)- β and bone morphogenetic protein (BMP). *Anat. Rec.* **258**, 119–127.
- Nieto, M. A., Sargent, M. G., Wilkinson, D. G., and Cooke, J. (1994). Control of cell behavior during vertebrate development by *slug*, a zinc finger gene. *Science* **264**, 835–839.
- Potts, J. D., and Runyan, R. B. (1989). Epithelial–mesenchymal cell transformation in the embryonic heart can be mediated, in part, by transforming growth factor β . *Dev. Biol.* **134**, 392–401.
- Potts, J. D., and Runyan, R. B. (1992). Sense and antisense TGF β 3 mRNA levels correlate with cardiac valve induction. *Dev. Dyn.* **193**, 340–345.
- Ramsdell, A. F., Moreno-Rodriguez, R. A., Wienecke, M. M., Sugi, Y., Turner, D. K., Mjaatvedt, C. H., and Markwald, R. R. (1998). Identification of an autocrine signaling pathway that amplifies induction of endocardial cushion tissue in the avian heart. *Acta Anat.* **162**, 1–15.
- Romano, L. A., and Runyan, R. B. (1999). *Slug* is a mediator of epithelial–mesenchymal cell transformation in the developing chicken heart. *Dev. Biol.* **212**, 243–254.
- Ros, M. A., Sefton, M., and Nieto, M. A. (1997). *Slug*, a zinc finger gene previously implicated in the early patterning of the mesoderm and neural crest, is also involved in chick limb development. *Development* **124**, 1821–1829.
- Runyan, R. B., and Markwald, R. R. (1983). Invasion of mesenchyme into three-dimensional collagen gels: A regional and temporal analysis of interaction in embryonic heart tissue. *Dev. Biol.* **95**, 108–114.
- Runyan, R. B., Potts, J. D., Sharma, R. V., Loeber, C. P., Chiang, J. J., and Bhalla, R. C. (1990). Signal transduction of a tissue interaction during embryonic heart development. *Cell Regul.* **1**, 301–303.
- Savagner, P., Yamada, K. M., and Thiery, J. P. (1997). The zinc-finger protein *Slug* causes desmosome dissociation, an initial and necessary step for growth factor-induced epithelial–mesenchymal transition. *J. Cell Biol.* **137**, 1403–1419.
- Sefton, M., Sanchez, S., and Nieto, M. A. (1998). Conserved and divergent roles for members of the Snail family of transcription factors in the chick and mouse embryo. *Development* **125**, 3111–3121.
- Selleck, M. A. J., Garcia-Castro, M. I., Artinger, K. B., and Bronner-Fraser, M. (1998). Effects of *Shh* and *Noggin* on neural crest formation demonstrate that BMP is required in the neural tube but not ectoderm. *Development* **125**, 4919–4930.
- Smith, S. M., Dickman, E. D., Power, S. C., and Lancman, J. (1998). Retinoids and their receptors in vertebrate embryogenesis. *J. Nutr. Suppl.* **128**, 467–470.
- Song, W., Majka, S. M., and McGuire, P. G. (1999). Hepatocyte growth factor expression in the developing myocardium: Evidence for a role in the regulation of the mesenchymal cell phenotype and urokinase expression. *Dev. Dyn.* **214**, 92–100.
- Wang, X.-F., Lin, H. Y., Ng-Eaton, E., Downward, J., Lodish, H. F., and Weinberg, R. A. (1991). Expression cloning and characterization of the TGF β Type III receptor. *Cell* **67**, 861–867.
- Yamagishi, T., Nakajima, Y., Miyazono, K., and Nakamura, H. (1999). Bone morphogenetic protein-2 acts synergistically with transforming growth factor- β 3 during endothelial–mesenchymal transformation in the developing chick heart. *J. Cell. Physiol.* **180**, 35–45.
- Yan, M., Nick, T. G., and Sinning, A. R. (2000). Retinoic acid inhibition of cardiac mesenchyme formation in vitro correlates with changes in the secretion of particulate matrix from the myocardium. *Anat. Rec.* **258**, 186–197.

Received for publication February 28, 2000

Revised April 12, 2000

Accepted April 12, 2000