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Endodermal growth factors promote endocardial precursor cell formation from precardiac mesoderm

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Abstract

We previously demonstrated that the initial emergence of endocardial precursor cells (endocardial angioblasts) occurred within the precardiac mesoderm and that the endodermal secretory products promoted delamination of cells from the precardiac mesoderm and expression of endothelial lineage markers [Dev. Biol. 175 (1996), 66]. In this study, we sought to extend our original study to the identification of candidate molecules derived from the endoderm that might have induced endocardial precursor cell formation. We have detected expression of transforming growth factors β (TGFβ) 2, 3, and 4 in anterior endoderm at Hamburger and Hamilton (H-H) stage 5 by RT-PCR. To address the role of growth factors known to be present in the endoderm, precardiac mesodermal explants were isolated from H-H stage 5 quail embryos and cultured on the surface of collagen gels with serum-free defined medium 199. Similar to the effect of explants cocultured with anterior endoderm, when cultured with TGFβs 1–3 (3 ng/ml each), explants formed QH-1 (anti-quail endothelial marker)-positive mesenchymal cells, which invaded the gel and expressed the extracellular marker, cytotactin (tenascin). Another member of the TGFβ superfamily, bone morphogenetic protein-2 (BMP-2; 100 ng/ml), did not induce QH-1-positive mesenchymal cell formation but promoted formation of an epithelial monolayer on the surface of the collagen gel; this monolayer did not express QH-1. Explants treated with vascular endothelial growth factor ($VEGF_{165}$, 100 ng/ml) also did not invade the gel but formed an epithelial-like outgrowth on the surface of the gel. However, this monolayer did express the QH-1 marker. Fibroblast growth factor-2 (FGF-2; 250 ng/ml)-treated explants expressed QH-1 and exhibited separation of the cells on the surface of the gel. Finally, a combination of TGFβs and VEGF enhanced formation of QH-1-positive cord-like structures within the gel from mesenchyme that had previously invaded the gel. Luminization of the cords, however, was not clearly evident. These findings suggest that TGFβs, among the growth factors tested, mediate the initial step of endocardial formation, i.e., delamination of endothelial precursor cells from precardiac mesoderm, whereas VEGF may primarily effect early vasculogenesis (cord-like structure formation).

Keywords: Endocardium; Precardiac mesoderm; Endoderm; Transforming growth factor β; Vascular endothelial growth factor; Fibroblast growth factor; Bone morphogenetic protein; QH-1; Cytotactin (Tenascin)

Introduction

The primary heart tube in all vertebrates consists of inner and outer cardiac epithelial walls, endocardium, and myocardium. Evidence from in vivo tracing (Garcia-Martinez and Schoenwolf, 1993) and fate mapping experiments (Stalsberg and DeHaan, 1969) in early chick embryos has indicated that the endocardial and myocardial lineages may arise from the precardiac mesoderm. Retroviral-mediated lineage tracing studies have also indicated that both endocardial and myocardial precursor cells are present in the heart-forming regions of HH stage 4/5 chick embryos (Cohen-Gould and Mikawa, 1996). These findings confirm that at least one cohort of endocardial precursor cells derives from the precardiac mesoderm as initially suggested by Sabin (1920).

While considerable descriptive information has been obtained delineating the development of the endocardium, there have been few experiments that address the mecha-

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nisms regulating this process (for reviews, see Baldwin, 1996; Fishman and Chien, 1997; Lough and Sugi, 2000). During its initial formation, following gastrulation and until incorporation into a tubular heart, the precardiac mesoderm is continuously associated with anterior lateral plate endoderm. Ablation and/or transplantation studies have provided considerable evidence that the endoderm has an instructive or permissive role in myocardiogenesis (reviewed by Jacobson and Sater, 1988; Sugi and Lough, 1994; Nascone and Mercola, 1995; Schultheiss et al., 1995; Lough and Sugi, 2000), hematopoietic cell formation, and vascular endothelial cell differentiation (Wilt, 1965; Pardanaud and Dieterlen-Lievre, 1999). However, except for our previous study which indicated that anterior lateral plate endoderm promoted endocardial endothelial precursor cell formation from the precardiac mesoderm (Sugi and Markwald, 1996), there have been few studies into the origin and regulation of endocardiogenesis.

In this study, we sought to extend our original study to determine candidate molecules derived from the endoderm that might have mediated endodermal signaling on precardiac mesoderm. Because polypeptide growth factors frequently mediate embryonic induction, we sought to test the hypothesis that endodermally derived growth factors induce formation of endocardial precursor cells and their assembly into vascular structures. The selection of growth factors was based on their expression or presence in the endoderm at the appropriate developmental window to interact with precardiac mesoderm.

Among those factors selected were members of the transforming growth factor βs (TGFβs) known to be expressed in the endoderm at the mRNA level, i.e., TGFβ1 and β2 in mice (Dickson et al., 1993; Roelen et al., 1994), and TGFβ2, β3 (Jakowlew et al., 1994), and β4, a chick cognate of mammalian β1 (Jakowlew et al., 1992), in chick. Type II TGFβ receptor, which is critical and constitutively essential for all TGFβ signaling (Wrana et al., 1994), is expressed in embryonic avian endothelial cells (Brown et al., 1996, 1999b). Endoglin, an ancillary TGFβ receptor, has also been detected in endocardial endothelial cells (Vincent et al., 1998). The importance of TGFβ signaling in endothelial cell formation and differentiation has been emphasized by the mutation of the TGFβ type II receptor, which caused defects in yolk sac vasculature and hematopoiesis (Oshima et al., 1996). Similar defects were reported in endoglin mutant mice (Arthur et al., 2000) and were consistent with those seen in TGFβ1 ligand knock out mice (Dickson et al., 1995).

Another TGFβ superfamily member, bone morphogenetic protein-2 (BMP-2), is also expressed in the endoderm adjacent to precardiac mesoderm (Schultheiss et al., 1997; Andrée et al., 1998; Ehrman and Yutzey, 1999). Receptors for BMPs, e.g., activin receptor IIA (ActR-IIA) (Stern et al., 1995) and BMP receptor 1B (BMPR-IB) (Lough and Sugi, 2000), are expressed in precardiac mesoderm at the mRNA level. Thus, the role of BMP-2 in endocardiogenesis was also investigated in this study.

We have included vascular endothelial growth factor (VEGF) in this study since it also has been demonstrated to be expressed in the endoderm of mouse embryos (Breier et al., 1995) and quail embryos (Flamme et al., 1995). VEGF and its receptors, VEGFR-1 (Flt-2) and VEGFR-2 (Flk-1, also known as KDR in human), may have an important function in differentiation of the endothelial lineage (Breier et al., 1995; Shalaby et al., 1995; Fong et al., 1995). Targeted inactivation of the genes encoding VEGF and its receptors resulted in defective vascular development and early embryonic lethality (Fong et al., 1995; Shalaby et al., 1995; Carmeliet et al., 1996; Ferrara et al., 1996). Fibroblast growth factor-2 (FGF-2) protein and mRNA, also detected in early chick endoderm, (Parlow et al., 1991; Sugi et al., 1993), have been implicated to play an important role in endothelial differentiation through their receptors (Flamme and Risau, 1992; Krah et al., 1995, Friesel and Maciag, 1995; Baldwin et al., 1996). Finally, recent assays using beads soaked with exogenous FGF-2 implanted into the somitic mesoderm of avian embryos suggested a direct role for FGF-2 in vasculogenesis/angiogenesis (Cox and Poole, 2000; Poole et al., 2001).

To study the regulation of early endocardial formation, we have established a primary culture system which recapitulates endocardiogenesis from precardiac mesoderm using a collagen gel culture assay (Sugi and Markwald, 1996). Using this assay system, we now show that (1) TGFβs but not BMP-2 promoted endocardial precursor cell formation from precardiac mesoderm cultured on the collagen gel as evidenced by QH-1 (quail endothelial marker)-positive invasive mesenchymal cell formation and expression of the extracellular marker cytotactin (tenascin); (2) Neither VEGF nor FGF-2 promoted invasive mesenchymal cell formation. However, VEGF enhanced formation of a QH-1-positive epithelial monolayer from precardiac mesoderm; (3) FGF-2 induced only separation of the cells on the surface of the gels; (4) Combination of TGFβs and VEGF enhanced formation of cord-like structures of QH-1-positive cells within the collagen gel. These findings suggest that TGFβs can mediate endocardial precursor cell formation from mesoderm, i.e., the delamination of endothelial precursor cells from precardiac mesoderm and expression of endothelial marker protein, whereas VEGF primarily effects early vasculogenesis. Portions of this work were previously reported in an abstract form (Sugi and Markwald, 1998).

Materials and methods

Embryos

Fertilized eggs from Japanese quail (*Coturnix coturnix japonica*) were incubated in a humid atmosphere at 38°C. Stages of embryonic development were determined by us-

ing the criteria made for chick embryos by Hamburger and Hamilton (1951).

Detection of transforming growth factor expression from endoderm by reverse transcription–polymerase chain reaction (RT-PCR)

The expression of TGFβs was detected by RT-PCR. This assay was employed because endoderm from the cardiogenic region in each Hamburger and Hamilton (H-H) stage 5 quail embryo contained approximately 4000–5000 cells.

Total RNA from H-H stage 5 endoderm was purified by using RNAzol (Tel-Test, Inc.; Chomczynski and Sacchi, 1987). Complementary DNA was prepared by using oligo-dT-primed MMLV reverse transcriptase (Promega). The cDNA was amplified by using *Thermus aquaticus (Taq)* DNA polymerase with 30 cycles of denaturation (94°C, 1 min), primer annealing (55°C, 1.5 min), and extension (72°C, 2 min). Primers were carefully designed to chicken TGFβ-2 (Jakowlew et al., 1990), chicken TGFβ-3 (Jakowlew et al., 1988a), and chicken TGFβ-4 (Jakowlew et al., 1988b). The primer pair for TGFβ2 was 5′-CAT-CTC-CAT-CTA-CAA-CAG-CAC-C-3′ (forward primer) and 5′-GCA-TTT-TTC-TCC-ATC-GCC-3′ (reverse primer). The primer pair for TGFβ3 was 5′-AGG-AGA-TGG-AGG-AGG-AGA-AG-3′ (forward primer) and 5′-AAG-CGG-AAC-ACA-TTG-GAG-3′ (reverse primer). The primer pair for TGFβ4 was 5′-ATG-AGT-ATT-GGG-CCA-AAG-3′ (forward primer) and 5′-ACG-TTG-AAC-ACG-AAG-AAG-3′ (reverse primer). That the predicted PCR products, 219 bp of TGFβ-2, 160 bp of TGFβ-3, and 109 bp of TGFβ-4, were not amplified from genomic DNA was verified by treating samples with RNase-free DNase-1 (Promega) before reverse transcription. As a positive control for the PCR, a 241-bp sequence of chick cytoplasmic β-actin was also amplified by using primers prepared according to the sequence published by Kost et al. (1983). The primer pair for cytoplasmic actin was 5′-GTA-CTC-TGT-CTG-GAT-TGG-3′ (forward primer) and 5′-TAA-TCC-TGA-GTC-AAG-CGC-3′ (reverse primer). All of the primers were purchased from Life Technologies (New York, NY). After the purification by QIAquick spin columns (QIAGEN), the PCR products were verified by thermal cycle sequencing using Taq DNA polymerase and fluorescent dye-labeled terminations (Medical University of South Carolina, Biotechnology Resource Laboratory).

Serum-free culture of cardiogenic mesoderm

Microdissection and culturing procedure have previously been described in detail (Sugi et al., 1993; Sugi and Lough, 1994; Sugi and Markwald, 1996). Briefly, stage 5 quail embryos were treated with 1 mg/ml collagenase/dispase (Boehringer-Mannheim, Indianapolis), in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) to loosen the connections between germ layers. Enzyme reaction was stopped by the 0.02% EDTA/PBS. Serum was not used in any phase of tissue isolation or culturing. Sheets of anterior lateral plate mesoderm, corresponding to the endocardial fate map (Stalsberg and DeHaan, 1969) shown in Fig. 1, were microdissected with tungsten needles. Each explant was plated on a hydrated collagen gel containing fibronectin (1.0 mg/ml rat tail tendon type I collagen, Collaborative Research, and 10 μg/ml bovine plasma fibronectin, Sigma). After attachment of the explants to the surface of the collagen gel, serum-free defined medium M 199 (GIBCO/BRL) supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenium (ITS, Collaborative Research), 100 unit/ml penicillin and 100 μg/ml streptomycin (SFM) was added and changed daily. For some experiments, to produce endoderm-conditioned medium, anterior lateral plate endoderm was explanted on the surface of a Coster nucleopore filter (Transwell, 0.4-μm pore size, Costar, Cambridge, MA) that was overlaid on a collagen gel as detailed in Sugi and Markwald (1996). The depth of the Costar apparatus provided a final 1.0-mm space filled with culture medium between endoderm and mesoderm. Therefore, in the Costar system, there was no direct attachment of endoderm cultured on the top of the filter and the mesoderm cultured on the surface of the gel and soluble factors secreted by the endoderm could go through the 0.4-μm pore.

TGFβs, FGF-2, and $VEGF_{165}$ were purchased from R&D Systems. Recombinant human BMP-2 was kindly provided by Genetics Institute (Cambridge, MA). SFM including either porcine TGFβ-1, β-2, and β-3 (3 ng/ml each); recombinant human FGF-2 (250 ng/ml); recombinant human $VEGF_{165}$ (100 ng/ml); or BMP-2 (100 ng/ml) was added individually or in combination after the explants attached to the collagen gel (2 h). Media, including growth factors, were changed daily, and morphological analysis of cultured mesoderm was performed every 24 h by using an inverted microscope equipped with Hoffman Optics (Olympus, IMT-2). Cultured explants were scored positive for invasive mesenchymal cell formation based on detection of at least five cells underneath the surface of the collagen gel on day three of culture.

Immunostaining of cultured explants on the collagen gel

Cultured explants in the collagen gel were rinsed with phosphate-buffered saline (PBS) and fixed with 100% cold (−20°C) methanol, then rehydrated through a graded methanol series. After a brief wash with distilled water, samples were rinsed with PBS and incubated with 10% normal goat serum in 1% bovine serum albumin (BSA)–PBS to block nonspecific binding for 1 h at 4°C. For double immunostaining, MF20 immunostaining was performed first at 4°C overnight. After rinsing with 1% BSA–PBS, the samples were incubated with RITC-labeled goat anti-mouse IgG (Organon Teknika Corp.), which was followed by 10% normal goat serum/1% BSA–PBS for blocking. The samples were then stained with either QH-1 or antibodies to

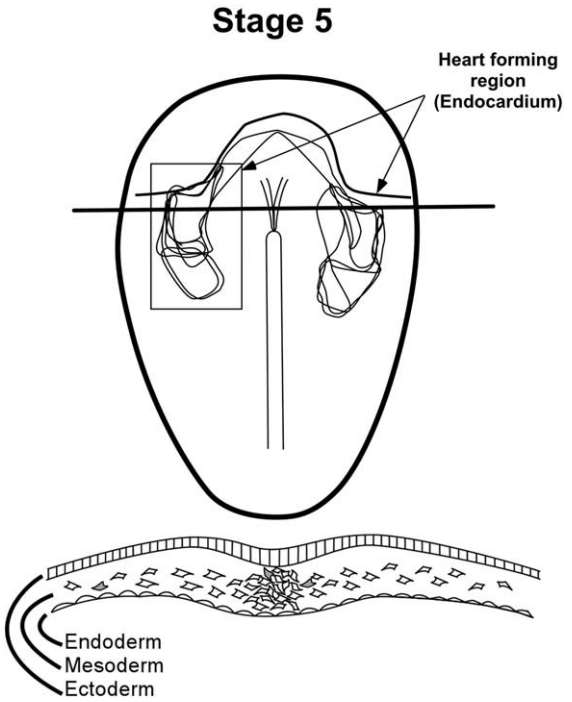


Fig. 1. Source of precardiac mesodermal explants. Heart-forming mesoderm was microdissected from anterior lateral plate of a stage 5 quail embryo based on stage 5 chick endocardial fate map (lines indicate each segment of the heart at stage 12, after Stalsberg and DeHaan, 1969). Diagram shows a transverse section at the level of the heart-forming region at stage 5, illustrating the germ layers used for the culture assays.

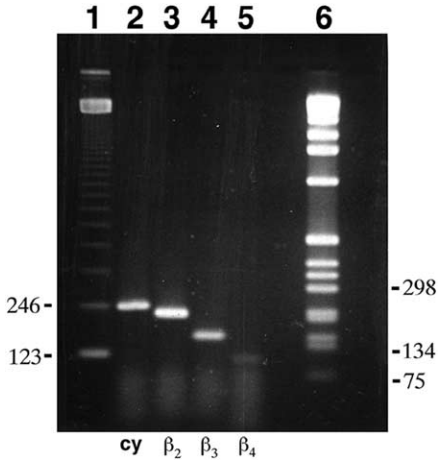


Fig. 2. Expression of TGFβs-2, 3, and 4 mRNA was detected by RT-PCR from stage 5 quail endoderm. Total RNA was purified and reverse transcribed, and the resultant cDNA was amplified by using primers that specify 219 bp of TGFβ2 (β2), 160 bp of TGFβ3 (β3), 109 bp of TGFβ4 cDNA, and, as a positive control for PCR, 245-bp sequence of cytoplasmic β-actin (cy). Lanes 1 and 6, respectively, contained 123-bp and 1-kbp ladder size markers. The PCR products were verified by thermal cycle sequencing.

cytotactin (generous gift from Dr. Stanley Hoffman, Medical University of South Carolina) followed by an incubation with FITC-labeled antibodies, goat anti-rabbit IgG for cytotactin, and goat anti-mouse IgG for QH-1. Samples were rinsed and mounted with DABCO (Sigma)/90% glycerol in PBS. Immunostained samples were observed under a Zeiss Axioskope fluorescent microscope.

Results

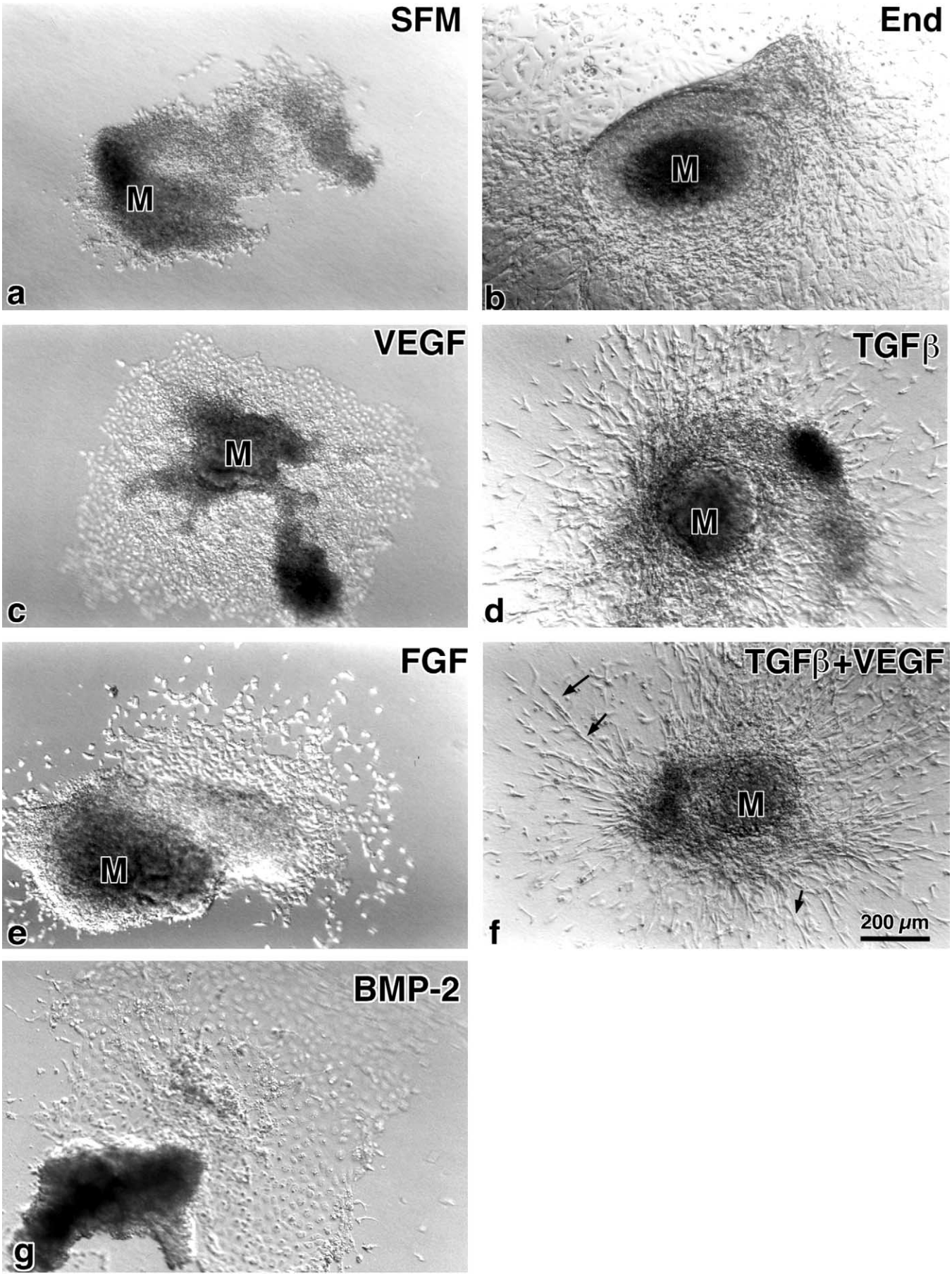
TGFβs are expressed in the heart forming endoderm

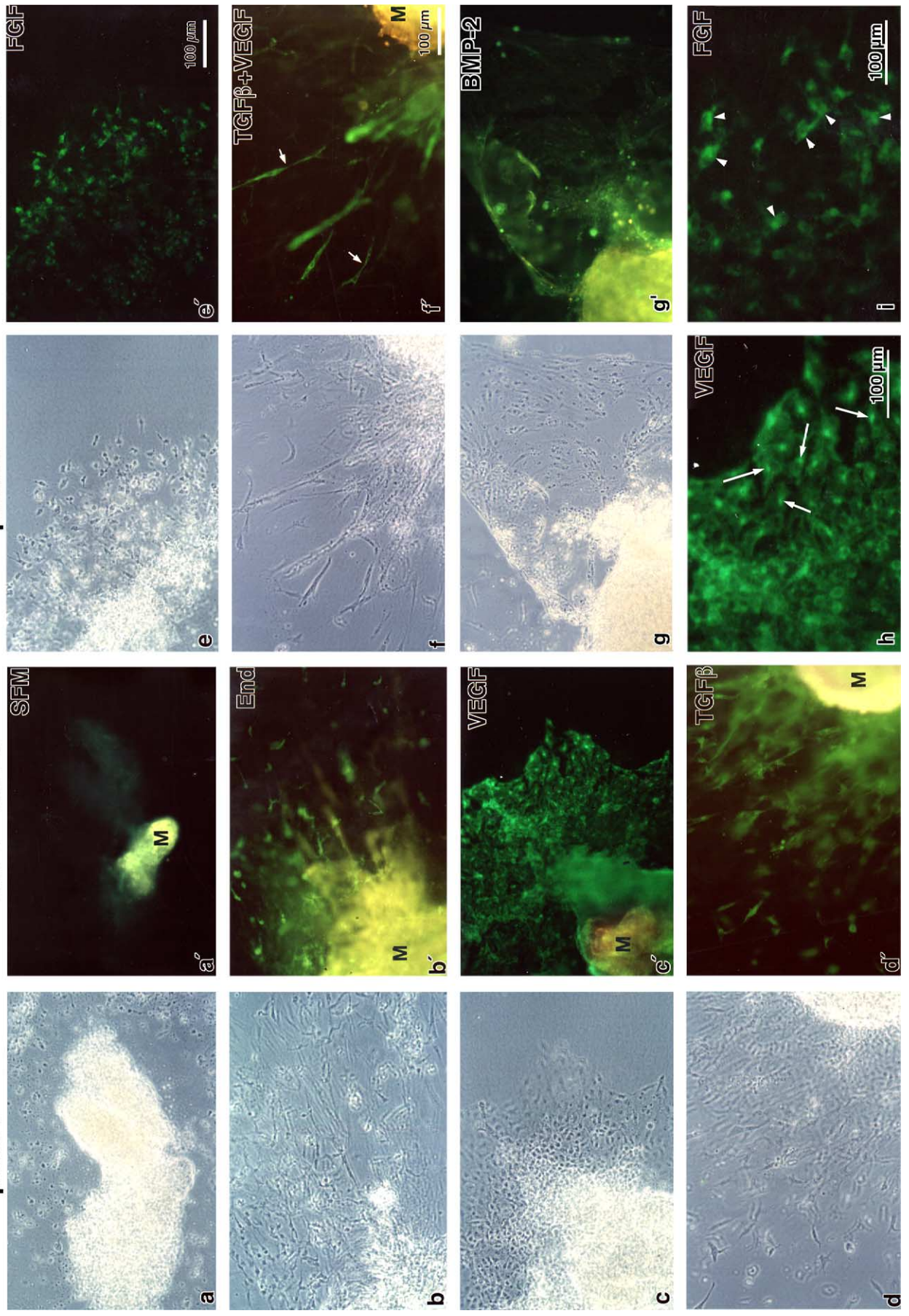
To evaluate the expression of TGFβs in heart-forming anterior lateral plate endoderm, endodermal tissue from stage 5 embryos (Fig. 1) was microdissected and applied for RT-PCR. Messenger RNAs for TGFβ2, β3, and β4 were detected from stage 5 endoderm (Fig. 2). These results confirm that the expected inducer, anterior lateral plate endoderm, is the source of TGFβs. Semiquantitatively, Fig. 2 indicates that relatively high levels of TGFβ2 and β3 are expressed in the anterior lateral plate endoderm.

Morphological effects of TGFβs, BMP-2, FGF-2, and VEGF

Our previous data indicate that endocardial precursor cells are derived, at least in part, from the precardiac mesoderm. At stage 5, the precardiac mesoderm is composed of two to three cell layers of undifferentiated cells that have no detectable characteristics of either myocardial or endothelial lineages, e.g., neither the endocardial marker QH-1 nor the myocardial marker MF20 has been detected. Using collagen gels and explants of stage 5 precardiac mesoderm, we sought to determine whether any of the selected growth factors known to be present in endoderm could simulate the effect of endodermal tissue on early endocardial formation.

Fig. 3. Morphological effects of endoderm-conditioned medium or growth factors on precardiac mesodermal explants from stage 5 quail embryos cultured on the surface of collagen gels. Mesodermal explants from stage 5 quail embryos were cultured on the surface of collagen gels in (a) serum-free-defined medium M199 supplemented with ITS alone (SFM), (b) endoderm-conditioned medium, (c) $VEGF_{165}$, (d) TGFβs-1, 2, and 3, (e) FGF-2, (f) TGF βs and VEGF, or (g) BMP-2. (a) Mesoderm cultured with SFM was smaller in size, stayed on the surface of the gel, and exhibited no invasion. (b) Endoderm-conditioned medium promoted invasive mesenchymal cell formation from the mesoderm. (c) VEGF-treated mesoderm formed an epithelial monolayer on the surface of the gel. (d) Mesoderm treated with TGFβs formed mesenchymal cells which invaded collagen gels. (e) FGF-2 caused only separation of the mesodermal cells on the surface of the gel. (f) Combination of TGFβs and VEGF enhanced formation of cord-like structures from invaded mesenchymal cells (arrows). (g) BMP-2-treated mesoderm formed only an epithelial monolayer on the surface of the gel. In each of the above treatments, contractile tissue was always formed as a three dimensional mass of the cells on the surface of the gel (M). Explants were photographed by using Hoffman optics. Scale bars, 200 μm.


SFM
M
a
End
M
b
VEGF
M
c
TGFβ
M
d
FGF
M
e
TGFβ+VEGF
M
f
200 μm
BMP-2
g


phase
QH-1 - MF20
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QH-1 - MF20
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BMP-2
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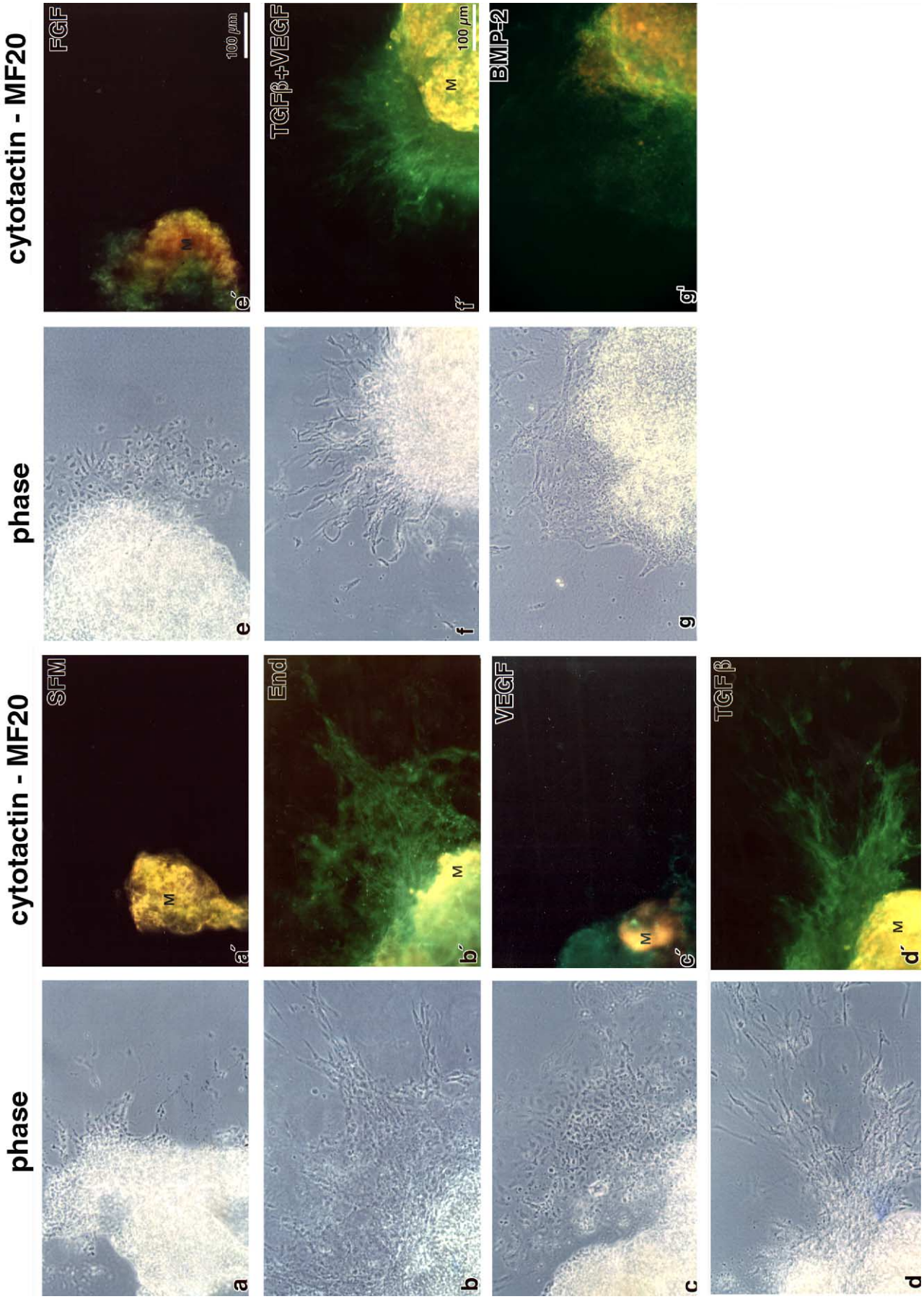
Figure 4


cytotactin - MF20
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End
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100 μm
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Figure 5

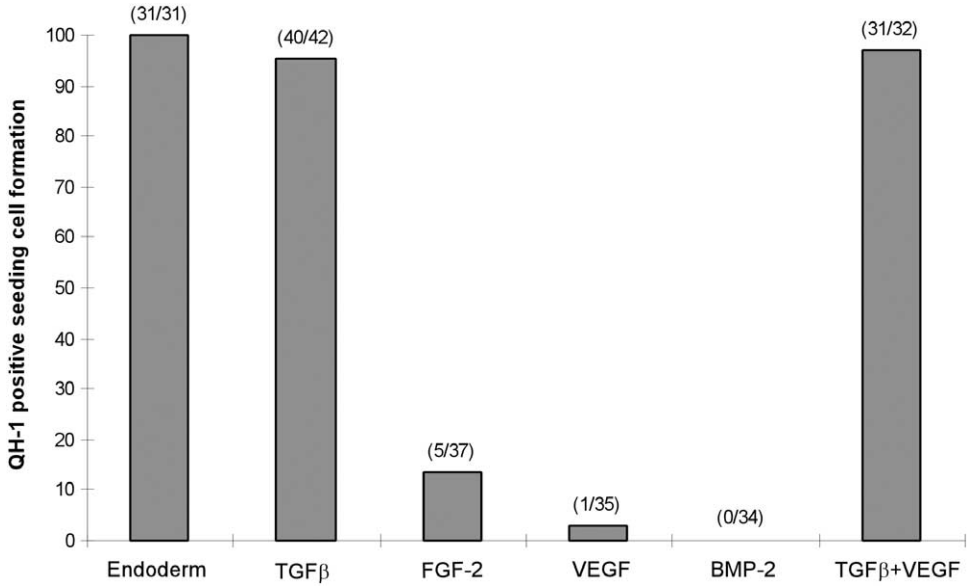

Fig. 6. Quantitative summary of invasive mesenchymal cell formation in growth factor-treated precardiac mesoderm. Stage 5 quail precardiac mesodermal explants were cultured with serum-free-defined medium M199 (SFM). Cultured explants were scored positive for invasive mesenchymal cell formation based on detection of more than five cells beneath the plane of the collagen gel surface. When cultured with TGFβ1–3 or TGFβ1–3 +VEGF, similar to the effect of endoderm-conditioned medium, invasive mesenchymal cell formation was evident. By contrast, FGF-2, BMP-2, and VEGF individually did not support invasive mesenchymal cell formation. The number in parenthesis indicates the number of explants that were evaluated.

When treated with endodermal conditioned medium, which is produced by the Costar Transwell apparatus (see details in Materials and methods), many mesodermal cells invaded the collagen gel, becoming free mesenchymal cells (Fig. 3b). Conversely, serum-free-defined medium supplemented only with ITS (SFM) failed to induce invasive mesenchymal cell formation from the mesoderm, and aggregates of undifferentiated mesodermal cells remained on the surface of the gels (Fig. 3a). Similar to the effect of endodermal conditioned medium, TGFβ1, β2, and β3 (3 ng/ml each, porcine recombinant) treatment of mesoderm in the absence of endoderm resulted in both formation of a contractile mass of cells on the surface of the gel, indicating myocardial differentiation, and free mesenchymal cells which invaded the collagen lattice (Fig. 3d), potentially representing early endocardial precursor cells. Importantly, when TGFβ1, β2, or β3 was added individually to the mesoderm singularly at a final concentration of 3 ng/ml, the same effects were found, including formation of free mesenchymal cells that invaded the collagen gel (data not shown). However, another TGF superfamily member, BMP-2 (100 ng/ml), was unable to induce invasive mesenchymal cell formation but

Fig. 4. MF20 and QH-1 double immunostaining of precardiac mesodermal explants from stage 5 quail embryos cultured on the surface of collagen gels. Phase contrast (a, b, c, d, e, f, g) and QH-1 (FITC, green color), MF20 (RITC, reddish yellow color) double immunofluorescence (a′, b′, c′, d′, e′, f′, g′, h, i) microscopy of mesodermal explants. (a′) Mesoderm cultured with SFM did not express QH-1. (b′) When cultured with endoderm-conditioned medium, mesenchymal cells invaded into collagen gels and expressed QH-1 antigen. (c′) VEGF-treated mesoderm exhibited small perinuclear QH-1 staining within the epithelial monolayer that formed on the surface of the gel but mesenchymal cell formation did not occur. (d′) By contrast, the mesoderm cultured with TGFβs formed QH-1-positive mesenchymal cells which invaded the gel lattice. (e′) FGF-2-treated mesoderm did exhibit QH-1 staining in cells that separated from each other. These cells did not invade the gel lattice. (f′) In mesoderm treated with TGFβs + VEGF, QH-1-positive cord-like vascular structures were formed within the gel (arrows). (g′) BMP-2-treated mesodermal epithelial monolayers did not express QH-1. Note the difference between epithelial cells treated with VEGF and BMP-2. In each of the above, MF20-positive myocardial cells were formed as a three-dimensional mass of the cells on the surface of the collagen (M). An MF20-positive contractile mass of the cells is always formed after the treatment of FGF-2. However, the particular sample presented in Fig. 4e′ lost the mass of the cells which grew on the surface of the gel during the whole-mount immunohistochemistry procedure. (h) Higher magnification view of (c′). This figure clearly indicates punctate perinuclear QH-1 staining within the VEGF-treated mesodermal cells (arrows). (i) Unlike VEGF-treated mesodermal cells, FGF-treated mesodermal cells showed more broad cytoplasmic-staining (arrow heads) similar to that observed in the cells treated with endoderm-conditioned medium or TGFβs. Scale bar, 100 μm.

Fig. 5. MF20 and cytotactin double immunostaining of precardiac mesodermal explants from stage 5 quail embryos cultured on the surface of collagen gels. Phase contrast (a, b, c, d, e, f, g) and cytotactin (FITC, green color), MF20 (RITC, reddish yellow color) double immunofluorescence (a′, b′, c′, d′, e′, f′, g′) microscopy of mesodermal explants. Mesoderm cultured alone in SFM remained epithelial and did not express cytotactin (a′). Cytotactin staining was observed only after treatments that induced invasive mesenchymal cell formation, i.e., endoderm-conditioned medium (b′), TGFβs (d′), or TGFβ + VEGF (f′), but not VEGF (c′), FGF-2 (e′), or BMP-2 (g′). In each of the above experiments, MF20-positive myocardial cells were formed as three-dimensional mass of the cells on the surface of the gel (M). Scale bar, 100 μm.

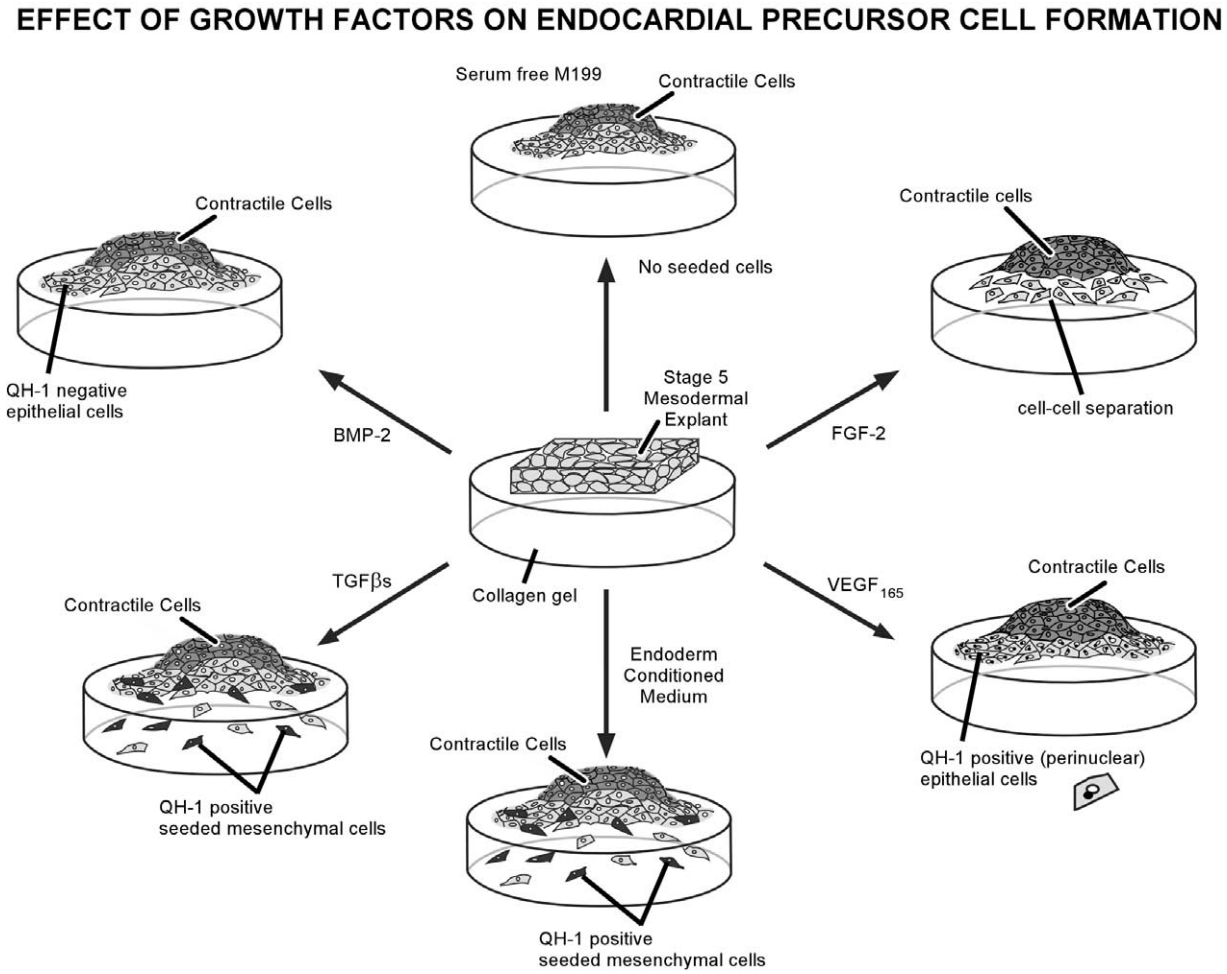

Fig. 7. Summary diagram of culture assays using stage 5 quail mesoderm. Precardiac mesoderm treated with endoderm-conditioned medium (bottom center) differentiated into contractile mass of cells on the surface of the collagen gel and numerous mesenchymal cells invaded the gel and expressed endothelial marker QH-1 and the mesenchymal marker, cytotactin. Conversely, mesodermal explants cultured in serum-free M199 (SFM) alone (top center) remained on the surface of the gels and expressed little, if any QH-1. TGFβ-treated explants resemble that of endoderm-conditioned medium treated, i.e., QH-1 expression and invasive mesenchymal cell formation. VEGF-treated explants formed epithelial monolayer on the surface of the gel, which express perinuclear cytoplasmic staining of QH-1. BMP-2-treated explants formed QH-1-negative epithelial monolayer on the surface of the collagen gel. FGF-2-treated explants exhibited only separation of the cells on the surface of the gel but no invasion of cells. Cytotactin expression was detected only after the treatment of endoderm-conditioned medium, TGFβs and TGFβ + VEGF, similar to the effect of endoderm. By contrast, FGF, VEGF, or BMP-2-treated explants did not express cytotactin. In each of the treatments, MF-20-positive myocardial cells were formed as a three-dimensional mass of cells on the surface of the gel presumably because of the insulin supplemented in the medium.

did promote the outgrowth of an epithelial cell monolayer on the surface of the collagen gel (Fig. 3g). Precardiac mesodermal explants treated with $VEGF_{165}$ (100 ng/ml) also did not give rise to cells that invaded into the gel but rather to cells that formed an epithelial monolayer on the surface of the gel (Fig. 3c). FGF-2 (200 ng/ml)-treated mesodermal explants formed a loose surface monolayer in which cells were partially separated from one another but did not from invasive mesenchymal cells (Fig. 3e). Thus, only TGFβ treatment was effective in promotion of invasive cells from a stage 5 mesodermal explant. When TGFβs were combined with VEGF, the cells that invaded the gel formed cord-like (vascular) structures within the gel lattice (Fig. 3f), which could not be enhanced or diminished by addition of either FGF-2 or BMP-2.

Endothelial marker, QH-1 expression in growth factor-treated precardiac mesoderm

At the onset of culture, stage 5 mesoderm does not express a QH-1 endothelial marker. In vivo findings (Coffin and Poole, 1988; Sugi and Markwald, 1996) indicate that QH-1 expression initially occurs in free mesenchymal cells associated with precardiac mesoderm at stage 7^{+}. In the present study, precardiac mesoderm cultured with SFM (supplemented only with ITS) formed an MF 20-positive, contractile mass of the cells on the surface of the gel, indicating myocardial differentiation, but neither invasive mesenchymal cell formation nor QH-1 expression was detected (Fig. 4a and a′). However, when cocultured with endoderm or endodermal conditioned medium, many cells

Table 1
Differential effect of growth factors on formation of endocardial precursors explanted from precardiac mesoderm at H-H stage 5

Expression phenotype	Treatment						
	Endoderm	SFM	TGFβs	BMP-2	FGF-2	VEGF	TGFβ +VEGF
MF20	+	+	+	+	+	+	+
QH-1	+	−	+	−	+	+[a]	+
Cytotactin	+	−	+	−	−	−	+
Cell separation	+	−	+	−	+	−	+
Cell seeding	+	−	+	−	−	−	+

[a] QH-1 staining in VEGF-treated mesoderm is perinuclear punctate staining.

migrated from the original explant, invaded the collagen gel, and expressed QH-1 (Fig. 4b and b′), in addition to formation of an MF20-positive contractile mass at the surface of the gels (M, yellow–red color in Fig. 4b′). Similar to the effect of endoderm, mesenchymal cells that invaded collagen gels in response to TGFβs also expressed a QH-1 endothelial marker (Fig. 4d and d′). Epithelial monolayers that formed in VEGF-treated precardiac mesoderm exhibited a perinuclear punctate staining pattern for QH-1 (Fig. 4c, c′, and arrows in Fig. 4h). This punctate staining pattern is distinctively different from the uniform staining pattern seen within invasive mesenchymal cells derived from the mesodermal explants treated with endoderm-conditioned medium or TGFβs. By contrast, the monolayer that formed in mesodermal explants treated with BMP-2 were QH-1-negative (Fig. 4g and g′), whereas FGF-2-treated mesodermal cells, which were separated but stayed on the surface of the gel, were QH-1-positive and showed similar cytoplasmic staining pattern to that of mesodermal cells treated with endoderm-conditioned medium or TGFβs. A punctate staining pattern which was seen in the cells treated with VEGF was not observed in FGF-treated cells (Fig. 4e, and e′, and arrowheads in Fig. 4i). All of the above-mentioned stage 5 mesodermal explants, regardless of the type of treatment, including just SFM, formed contractile masses on the surface of the collagen gel that expressed MF20 (M, yellow–red color in Fig. 4a′, b′, c′, d′, f′, and g′), a myocardial differentiation marker protein. As Fig. 5e′ indicates, an MF20-positive contractile mass of the cells is always formed after the treatment of FGF-2. Myocardial differentiation in the SFM was presumably supported by ITS (Sugi and Lough, 1995).

Cytotactin (tenascin) expression in growth factor treated precardiac mesoderm

Cytotactin is one of several mesenchymal marker proteins expressed during early cardiac formation (Crossin et al., 1986; Sugi and Markwald, 1996). Intense cytotactin expression is revealed in the mesenchymal precursors of the endocardium from stage 7^+ to stage 9. However, its expression is lost after mesenchymal cells become epithelialized to form a definitive endocardial tube (Sugi and Markwald, 1996). Using cytotactin as a marker for mesenchymal cell formation, we found that TGFβs or TGFβ + VEGF treatment induced cytotactin expression within invasive mesenchymal cells, which was similar to the effect of endoderm (Fig. 5b, b′, d, d′, f, and f′). By contrast, when added individually, FGF-2-, VEGF-, or BMP-2-treated explants did not express cytotactin up to 7 days of culture (Fig. 5a, a′, c, c′, e, e′, g, and g′). In each of the above experiments, the three-dimensional aggregates of contractile cells that had grown on the surface of the gel were MF20-positive (M, yellow–red color in Fig. 5a′, b′, c′, d′, e′, f′, and g′).

Combination of TGFβs and VEGF promote assembly of preendothelial cells and formation of cord-like structures

When added in combination to the mesodermal explants, VEGF and TGFβs enhanced cell-to-cell association of invaded QH-1-positive mesenchymal cells and the formation of QH-1-positive cord-like structures (Figs. 3f, and 4f′). Such cord-like structure formation is typically seen in vivo during early vasculogenesis. However, lumen formation was not clearly evident in serial sections of paraffin embedded mesodermal explants. These results are summarized in Figs. 6 and 7, and Table 1.

Discussion

Endocardial precursor cell formation from precardiac mesoderm

The differentiation process of endocardial precursor cells from precardiac mesoderm has been well documented by Linask's group (Linask, 1992; Linask and Lash, 1993; Linask et al., 1997). According to their description, at H-H stage 5, precardiac mesoderm expresses the adhesion molecule N-cadherin evenly on all cell surfaces. During coelom formation at around H-H stage 7–8, myocardial precursor cells change shape and show a distinct polarity conveyed by the apical expression of N-cadherin, lateral expression of Na^+ K^+ ATPase, and enrichment of integrin on the basal cell surface, indicating acquisition of an epithelial nature. The endocardial precursor cells reside close to the endoderm and eventually downregulate N-cadherin, which

correlated with endocardial cell sorting and aggregation (Linask, 1992). Also, in culture assay experiments, Linask and Lash (1993) described that the loss of N-cadherin expression by endocardial cells accompanied differentiation and their eventual dissociation from N-cadherin-positive myocardiocytes in mesodermal explants (Linask and Lash, 1993). In support of their finding, we found that stage 5 precardiac mesoderm did not express any endothelial (QH-1) or myocardial (MF20) markers and expressed mesenchymal markers (cytotactin or JB-3 antigen, fibrillin-2) weekly at H-H stage 5 (Sugi and Markwald, 1996). QH-1 expression and strong mesenchymal marker expression was detected in the heart-forming region after H-H stage 7+ (Sugi and Markwald, 1996). Taken together, these findings suggest that, at H-H stage 5, the precardiac mesoderm is undifferentiated, i.e., it expresses N-cadherin uniformly on all cell surfaces, does not express any myocardial or endocardial marker protein, and only weakly expresses mesenchymal marker proteins. However, after the onset of the coelom formation at H-H stage 7+−8, precardiac mesoderm differentiates into (1) N-cadherin-positive-epithelialized myocardial precursor cells in the splanchnic mesoderm, which eventually express myocardial markers and (2) endocardial precursor cells, which lose expression of N-cadherin, delaminate from the future splanchnic mesoderm by invading between the interface of endoderm and mesoderm, and express endothelial (QH-1) and mesenchymal (cytotactin and Fibrillin-2) markers. Upon examination of transverse sections of paraffin-embedded chick embryos and collagen gel cultured explants, undifferentiated precardiac mesodermal cells at stage 5 appear to be more cuboidal, whereas endocardial precursor cells at stage 8 appear to be more elongated and invasive (Sugi et al., 1995; Sugi and Markwald, 1996). Thus, the endocardial precursor cell formation from precardiac mesoderm is associated with a change of cell shape and expression of marker proteins. In the present study, by using a collagen gel culture system, we have revealed that endoderm-conditioned medium or TGFβs induced this phenotypical change in mesodermal explants, i.e., acquisition of invasive mesenchymal phenotype and expression of QH-1 endothelial and cytotactin mesenchymal markers.

Endocardial precursor cell formation is recapitulated in collagen gel cultured precardiac mesoderm

In summary, we propose that the following developmental progression occurs during formation of endocardium. Epiblast cells that ultimately form endocardium and myocardium migrate during stage 3 through the rostal half of the primitive streak (Garcia-Martinez and Schoenwolf, 1993) to form paired cohorts of cells at stage 5 within the anterior lateral plate mesoderm, known as precardiac mesoderm (Rawles, 1943; Rosenquist and DeHaan, 1966; Stalsberg and DeHaan, 1969; Redkar et al., 2001). Thereafter, endocardial precursors are segregated from myocardial precursors, with the latter occupying the epithelial layer of the splanchnic mesoderm by stage 7–8 while the former invade the endoderm–mesoderm interface, becoming free invasive mesenchymal cells which express both endothelial and mesenchymal markers (Linask, 1992; Linask and Lash, 1993; Sugi and Markwald, 1996; Linask et al., 1997). Subsequently, the mesenchymal endocardial precursor cells assemble into a cord-like plexus at stage 8–9 from which a definitive single epithelial tube of endocardium is directly formed by stage 10 (De Ruiter et al., 1992; Sugi and Markwald, 1996).

Importantly, all those characteristics of endocardiogenesis that occur in vivo, including (1) expression of the endothelial and mesenchymal marker proteins; (2) formation of invasive mesenchymal precursor cells; and (3) their assembly into vascular-like structures, are also recapitulated in the collagen gel culture system when treated with endoderm under appropriate serum-free conditions. Whereas, when treated with control serum-free M199 alone, the precardiac mesoderm remains as an aggregate on the surface of the collagen gels and does not express endothelial and mesenchymal marker proteins (Sugi and Markwald, 1996). In the present paper, we endeavor to extend these findings to the identification of candidate molecules which potentially mediate the endocardiogenic effects of endoderm on mesodermal explants.

TGFβs are expressed in the endoderm at the critical timing of endocardiogenesis and TGFβs induce initiation of endocardial precursor cell formation from mesoderm

In later stages of heart morphogenesis, the role of TGFβ signaling in endocardial cells has been well documented, particularly with regard to atrioventricular endothelial cell transformation to mesenchymal cells (Potts and Runyan, 1989; Brown et al., 1996, 1999a; Nakajima et al., 1994, 1998; Ramsdell and Markwald, 1997). However, the role of TGFβs in early endocardiogenesis has received little attention. The present findings are the first to indicate that TGFβs induce endocardial precursor cell formation from undifferentiated precardiac mesoderm.

Using RT-PCR, we found that TGFβ2, β3, and β4 (note that chick TGFβ4 is a homologue of mammalian TGFβ1) are expressed at mRNA level in isolated anterior lateral endoderm at stage 5 (Fig. 2), the time point at which endodermal induction of endocardial precursor cell formation is initiated in vivo (Sugi and Markwald, 1996). These findings correlate with the expression of the Type II TGFβ receptor, essential for all TGFβ signaling (Wrana et at., 1994). Type II TGFβ receptor expression has been reported in the developing endocardial endothelial cells (Barnett et al., 1994; Brown et al., 1999b), which suggests that the potential for TGFβ signaling exists during early endocardial formation. Therefore, we added TGFβ ligands to the culture medium to determine whether these growth factors have a regulatory role in endocardiogenesis.

Specifically, we observed that TGFβs (β1, β2, and β3) were potent stimulators of endocardial precursor cell formation as evidenced by the release of invasive mesenchymal cells from mesodermal explants and their concomitant expression of QH-1, as summarized in Fig. 7 and Table 1. We found that mixtures of recombinant porcine TGFβs (β1, β2, and β3) (Figs. 3, 4, and 5) as well as individually added TGF β1, β2, or β3 (data not shown) all promoted mesenchymal cell formation and QH-1 expression. Preliminary experiments performed prior to the present study indicated that invasive mesenchymal cell formation was elicited by TGFβs at concentrations as low as 0.5 ng/ml, i.e., 2/6 for TGFβ1, 2/4 for TGFβ-2, and 3/6 for TGFβ3. However, more stable induction was observed at the concentration of 3 ng/ml of each of the TGFβs: 5/5 for TGFβ1, 4/5 for TGFβ2, and 5/6 for TGFβ3. We did not observe significant differences in effects among TGFβs in QH-1-positive mesenchymal cell formation. This tendency remained the same when the concentration of TGFβs was increased to 10 ng/ml. Three ng/ml of TGFβs is considered to be a biological dosage and this concentration is lower than the level of TGFβs often used for transformation assays in endocardial cushion tissue formation, e.g., 50 ng/ml (Nakajima et al., 1994, 1998) or 10 ng/ml (Ramsdell and Markwald, 1997; Boyer et al., 1999). Examining the results collectively, it is unlikely that 3 ng/ml of TGFβs used in the present paper would cause over stimulation. The present findings suggest that signaling effects of TGF β1, β2, and β3 are conserved in their potential to induce formation of endocardial precursor cells from cultured explants of precardiac mesoderm. This notion is also consistent with the observation of possible redundancy in TGFβ knock out mice (Dickson et al., 1995; Sanford et al., 1997), which did not reveal prominent differences in endocardial formation. However, as endocardial cells differentiate, their response to TGFβ ligand appears to become more specific—as exemplified by the AV endocardium (Boyer et al., 1999). The present data, which indicate that TGFβs are potent mediators of endocardial precursor cell formation, are further supported by our recent results of inhibition of QH-1-positive invasive mesenchymal cell formation by adding antibodies to type II TGFβ receptor in endoderm–mesoderm coculture, which blocks all of TGFβ signaling (data not shown).

Regarding BMP-2, this member of TGFβ superfamily has also been reported to be expressed in the endoderm and implicated in myocardial differentiation from precardiac mesoderm (Lough et al., 1996; Schulthies et al., 1997; Andrée et al., 1998; Ehrman and Yutzey, 1999). BMP receptors ActR-IIA (Stern et al., 1995) and BMPR-IB (Lough and Sugi, 2000) are expressed in the precardiac mesoderm, supporting the idea that endodermally derived BMP-2 is involved in early heart formation. However, our present data revealed distinct differences between the effects of TGFβs and BMPs in endocardial precursor cell formation. Differential effects of the TGFβs and BMP-2 have also been reported in myocardial differentiation in the epiblast and in the precardiac mesoderm (Ladd et al., 1998). Since BMP-2 did not support either expression of endothelial marker protein or formation of mesenchyme, it would suggest that, prior to formation of a definitive heart tube, the role of BMPs may be directed at promoting or sustaining myocardial cell formation and/differentiation (Lough et al., 1996; Schulthies et al., 1997; Andrée et al., 1998; Reiter et al., 2001).

VEGF is a potent stimulator of endothelial epithelium formation but not endocardial precursor mesenchymal cell formation

VEGF is another potent vasculogenic molecule, which we found to have a significant role in endocardiogenesis. As summarized in Fig. 7 and Table 1, one important finding was that exogenously added $VEGF_{165}$ did not induce mesenchymal cell formation but rather induced a monolayer of epithelial cells to grow out from precardiac mesoderm, which expressed perinuclear staining for the endothelial marker QH-1 (Figs. 3c, 4c′, and 4h). Most interestingly, when VEGF was added together with TGFβs, the mesodermal explants created cord-like, vascular structures within the three-dimensional framework of the collagen gels as occurs in vivo during formation of the endocardial tube (Sugi and Markwald, 1996). VEGF acts by stimulating members of a family of receptor tyrosine kinases that include VEGFR-2 and VEGFR-1. VEGFR-2 is the earliest receptor expressed in a subset of mouse (Yamaguchi et al., 1993) and quail mesoderm (Eichmann et al, 1998). VEGFR-1 is expressed in endothelial precursor cells in the mesoderm at slightly later stages and in endothelial cells that have formed vascular tube (Yamaguchi et al., 1993). Mice homozygous for VEGF gene disruption die by ED10.5 (Carmeliet et al., 1996) or ED11.5 (Ferrara et al., 1996). Disruption of the gene for VEGFR-2 seems to interfere with early formation of endothelial precursor cells of both blood vessels and hearts as well as hematopoietic cells, leading to early embryonic lethality (ED8.5–9.0) (Shalaby et al., 1995). Disruption of the gene for VEGFR-1 does not impair early formation of endothelial cells but seems to interfere with their later differentiation into vascular structures (Fong et al., 1995). These findings suggest that VEGF, acting through two different VEGF receptors, has two different developmental roles: one early in endothelial lineage determination/formation (angioblast formation) and one later in endothelial cell differentiation/endothelial lumen formation (vasculogenesis). In this context, the data we obtained from our collagen gel culture assays of precardiac mesoderm treated with $VEGF_{165}$ correlated closely with both potential roles for VEGF, e.g., inducing lineage determination (QH-1 expression) and later differentiation/lumen formation (epithelialization of angioblasts). Our culture data also compare favorably with that obtained by microinjection of $VEGF_{165}$ into parasomitic regions, which induced cell protrusive activity and fusion of preformed vascular structures called

"polygons" into larger vessels (Drake and Little, 1996; Drake et al., 2000). Based on our data, we would propose that VEGF mediates, in part, the effects of endoderm on endocardial vasculogenesis through mechanisms that promote a "reversed" epithelial–mesenchymal transformation in which free mesenchymal endocardial precursor cells (angioblasts) become epithelialized to form a definitive endocardially lined cardiac tube.

FGFs are thought to regulate endothelial marker protein expression in quail blasto disc cultures (Flame and Risau, 1992). Angioblast induction and vessel formation and patterning have also been reported when FGF protein-soaked beads were implanted into noncardiogenic somitic mesodermal regions of stage 10 quail embryos (Cox and Poole, 2000). Consistent with Flam and Risau (1992) and Cox and Poole (2000), our present study also indicated that FGF-2 induced endothelial marker QH-1 expression. However, Flame and Risau (1992) used blasto disc cultures at much earlier stages than those that we studied, and they did not address invasive mesenchymal cell formation, which is the initial step of endocardial precursor cell formation. Cox and Poole (2000) used stage 10 quail embryos in which endocardial precursor cells had already been formed and had assembled into the heart tube. Our culture assay system utilized stage 5 anterior lateral plate mesoderm that strictly adhered to the endocardial fate map (Fig. 1). Rigorous adherence to the fate map is essential as the heart field may not be fully defined by the expression of any known embryonic genes at stage 5 (Eisenberg, 2002). It must be emphasized that at stage 5 there are no detectable in vivo signs of endocardial precursor cells in terms of expression of endothelial/angioblast marker protein, QH-1. The initial expression of QH-1 marker protein is detected only after stage 7^+ (Coffin and Poole, 1988; Sugi and Markwald, 1996). Therefore, our experiments addressed regulation of the initial emergence of the endocardial precursor cells from undifferentiated mesoderm, while experiments conducted after the emergence of the angioblasts (Cox and Poole, 2000) addressed regulation of differentiation and/or proliferation of angioblasts already determined to be in an endothelial lineage. This is further suggested by Pardanaud and Lievre (1999) who indicated that endoderm and selected growth factors induced formation of hemangiopoetic cells in the associated mesoderm at stages 13–16, much later than the stage (stage 5) we studied in the present report. Our careful examination of each growth factor by using precardiac mesodermal explants from stage 5 quail embryos revealed that FGF-2 and VEGF induced expression of the endothelial marker, QH-1, but did not induce invasive mesenchymal cell formation, which is an essential and initial step of endocardial precursor cell formation. Conversely, TGFβs induced invasive mesenchymal formation and QH-1 endothelial marker expression. We conclude that our findings most likely address the initial steps of cardiac angioblast formation events.

Regarding lineage determination by the growth factors, it has not been made clear whether endocardial cells are derived from a separate lineage from cells that comprise the lining of the vascular endothelium and if different mechanisms are involved. Recent evidence indicates that endocardial endothelial cells are biochemically distinct from cells that comprise the endothelium of blood vessels. For example, expression of the transcription factor, nuclear factor of activated T cells (NFATc1), is restricted to endocardial endothelial cells and that NFATc1 mRNA in murine precardiac mesoderm at ED 7.5 (de la Pompa et al., 1998) may mark cells specified to an endocardial lineage. NFATc1 null mice fail to develop normal cardiac valves and septa, structures that contain the progeny of endocardial endothelial cells and die at ED 13.5 (de la Pompa, 1998; Ranger et al., 1998). NFATc3 and NFATc4 mutant mice die around ED11 with generalized defects of vessel assembly as well as excessive and disorganized growth of vessels, which closely resemble the phenotype of calcineurin B (CnB) mutant mice (Graef et al., 2001), while NFATc1 mutant mice have no discernible defects in angiogenesis (de la Pompa, 1998; Ranger et al., 1998). These data suggest different roles for each NFATc gene in endocardiogenesis and vessel angiogenesis/vasculogenesis and further suggest the possibility that endocardial and vessel endothelial cells are regulated through different molecular pathways. Other evidence that endocardial and vascular endothelial cells are distinct is provided by the zebrafish mutant *cloche*, which lacks an endocardial tube but not a blood vessel endothelium (Stainier et al., 1995); subsequent findings that *cloche* acts upstream of VEGFR-2 (Liao et a., 1997) further raise the possibility that the formation of endocardial and vascular endothelial cells is regulated by different signaling pathways. In this context, studies which address interactions between endodermal growth factors and endocardial specific factors like NFATc1 could shed further light on the mechanisms underlying early endocardiogenesis.

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