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Identification of Transferrin as One of Multiple EDTA-Extractable Extracellular Proteins Involved in Early Chick Heart Morphogenesis

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Abstract It was demonstrated previously that a polyclonal antibody (ES1) raised against EDTA extractable proteins from embryonic chicken heart blocks cardiac endothelial-mesenchymal transformation in a culture bioassay and stains extracellular matrix at sites of embryonic inductive interactions, e.g., developing heart, limb buds, and neural crest forming region [Krug et al., 1987, Dev Biol 120:348–355; Mjaatvedt et al., 1991, Dev Biol 145:219–230). In the present study, by using an antiserum (ES3) to a similar immunogen, we affinity purified four major EDTA-soluble proteins. These proteins migrated as 27, 44, 63, and 70 kD molecules under reduced conditions and 27, 41, 52, and 59 kD under nonreduced conditions, respectively, on SDS-PAGE. Based on several criteria, the protein migrating at 70/59 kD (reduced/nonreduced) was indistinguishable from chicken transferrin (conalbumin): 1) amino acid sequencing showed that eight N-terminal residues were identical to those of chicken transferrin, 2) acid hydrolysates of both proteins had nearly identical compositions, 3) the protein co-migrated exactly with chicken transferrin under both reduced and nonreduced conditions, and 4) ES3 IgG recognized both the 70/59 kD protein and chicken transferrin by western blot analysis of nonreduced samples, but not with reduced samples. Immunohistochemistry of chicken embryonic heart with antibodies against transferrin demonstrated that anti-transferrin immunoreactivity is present in myocardium but absent in cardiac endothelium before the initiation of cardiac endothelial-mesenchymal formation. However, both cardiac endothelium and migrating mesenchymal cells became immunoreactive with anti-transferrin at the time transformation occurred. These findings suggest a possible involvement of transferrin in the inductive process of cardiac endothelial-mesenchymal transformation.

Key words: EDTA soluble proteins, chick embryo, purification, epithelial-mesenchymal interactions, transferrin, conalbumin, extracellular matrix

Inductive tissue interactions governing cell determination and morphogenesis are mediated by hypothetical signal substances [reviewed in Jessell and Melton, 1992]. The molecular identification of such putative inductive signals and the molecular responses of their target cells is the subject of much current interest. As a model system, we have been analyzing an inductive interaction in the early embryonic heart that gives origin to a population of mesenchymal cells that establish the blueprint for remodeling the simple tubular heart into a four chambered organ. In this system, early myocardium induces competent cardiac endothelial cells in the atrioventricular canal (AV) to transform from an epithelial morphology into a migrating mesenchymal population. Alterations in the formation of endothelial-derived mesenchyme is linked to a large proportion of congenital heart abnormalities [Clark and Takao, 1990].

The transformation of endothelium into mesenchyme is a multistep process. Upon stimulation, competent endothelial cells up or down regulate genetic expression of several molecules, including transforming growth factor beta (TGF beta), a homeotic gene (*Hox*-7), and cell:cell and cell:substratum adhesion molecules [Robert et al., 1989; Mjaatvedt and Markwald, 1989; Crossin and Hoffman, 1991; Funderberg and Markwald, 1986; Akhurst et al., 1990]. We have shown

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that proteins present within the cardiac extracellular matrix separating the endothelium and myocardium can be extracted from this extracellular space by EDTA. The resulting extracts, which contain as little as 0.05 μg protein per heart, can induce the transformation of target cardiac endothelial cells into mesenchyme in 3D collagen gel cultures. Similarly, the conditioned growth medium from primary cardiocyte cultures will induce endothelial transformation to mesenchyme [Krug et al., 1987]. These data suggest that the transformation of AV endothelium is mediated by myocardially derived extracellular matrix. The identification and characterization of these putative signals would be a significant step towards understanding the mechanisms involved in this critical event in early cardiogenesis.

In order to approach this task we prepared a polyclonal antiserum (ES1) against EDTA soluble, extracellular proteins extracted from 800 embryonic chick hearts [Krug et al., 1987]. ES1 antibodies effectively blocked endothelial transformation to mesenchyme when either added directly to the culture system or by ES1 immunoadsorption of cardiocyte conditioned medium [Mjaatvedt et al., 1991]. When used for immunostaining, ES1 antibodies localized to the extracellular matrix of the heart; specifically, ES1 antigens correlated temporally and regionally with sites where endothelial cells transformed to mesenchyme. These data imply that ES antigens, either directly or indirectly, induce AV mesenchyme formation.

Interestingly, ES1 antigens were not restricted to just the heart but expression was also observed at other sites of known inductive interactions, including limb mesoderm and apical ectodermal ridge, the extracellular matrix associated with sites of neural crest formation, the notochord, and ventral floor of the neural tube [Mjaatvedt et al., 1991; Isokawa et al., 1991; Erickson, unpublished observations]. These and other data [Rezaee et al., 1993] suggest ES antigens may function globally as mediators of inductive tissue interactions.

Further characterization of ES antigens using these antibodies has proven difficult owing to the very limited supply of the antibodies. The purpose of the present study was to produce a second generation antiserum to ES antigens for subsequent immunopurification and characterization studies. Using newly generated antisera (ES2 and ES3) that immunohistochemically stained and blocked AV mesenchyme formation similarly to the original ES1, four major ES antigens were isolated. One of these, migrating at 70/59 kD (reduced/nonreduced), was found to be indistinguishable from chicken transferrin (conalbumin). Based on this identification, we compared the immunohistochemical distribution of transferrin to ES antigens in the embryonic heart. As the putative ES/70 antigen, the temporal and regional distribution of transferrin was consistent with its identification as an ES antigen. However, the pattern of expression for transferrin in the embryonic heart is more consistent with an autocrine rather than paracrine role for this particular ES antigen.

MATERIALS AND METHODS

Production of ES2/ES3 Polyclonal Antibodies

Owing to the mortality of our ES1 antibody supplier, it was necessary to generate a new source of antibodies against EDTA-extractable embryonic heart extracellular matrix. Immunogen was prepared in a similar method to that described previously for ES1 antibody production [Krug et al., 1987]. Briefly, 2,000 hearts were collected from stage 14–16 chicken embryos [Hamburger and Hamilton, 1951], rinsed ten times briefly, then three times (10 min each) with ice-cold Earle's balanced salt solution (EBSS), and then extracted with phosphate-buffered saline (PBS), containing 5 mM EDTA, 1 mM phenylmethylsulfonylfluoride (PMSF), 1 μM leupeptin, and 1 μM pepstatin A, for 60 min at 4°C by gentle inversion mixing. Following extraction, intact heart tissue was removed by centrifugation at 1,500*g* for 5 min. The resulting supernatant was made 10 mM in $CaCl_2$ and incubated for 10 min at 4°C to encourage any divalent mediated interactions of proteins and then centrifuged (100,000*g*; Beckman Type 50.2 Ti rotor at 32,000 rpm) for 120 min at 4°C. The supernatant fraction was concentrated by ultrafiltration (Centriprep-10, 10,000 molecular weight cutoff; Amicon, MA) and served as immunogen for ES3 antibody production. The pellet fraction was washed, recentrifuged (100,000*g*), resuspended in PBS, and used as immunogen for ES2 antibody production. These two preparations of immunogen were emulsified separately (1:1, v/v) with Freund's complete adjuvant and one third of the final volume injected intradermally into multiple sites on the back of two New

Zealand rabbits. Three and six weeks later, the animals were boosted with one-third volumes of the remaining emulsions. Protein G affinity chromatography (AbSorbent G; Genex, MD) was used to isolate the IgG fraction from sera collected 2 weeks after the second booter injection.

Preparation of Immunoaffinity Disks

For small scale pilot purification with ES2 and ES3, 700 μl of either ES2- or ES3-IgG (2.0 mg/ml) in 0.5 M carbonate buffer (pH 9.3) was loaded to a preactivated affinity disk support (U12 disk unit; Nalgene) and incubated overnight at room temperature. The disks were stabilized with 0.1% sodium borohydride in PBS and rinsed sequentially with 15 ml each of PBS, 1 M NaCl, 0.1 M glycine buffer (pH 2.3), and PBS. Approximately 500 μg of IgG was bound (33% binding efficiency).

ES3 was selected as a ligand for large scale purification of antigens as described in Results. Four milliliters of ES3-IgG (2.4 mg/ml) was allowed to absorb into 12 preactivated membrane disks (47 mm MAC active discs; Amicon, MA). Membranes were dried in a desiccated container overnight at 4°C and mounted in a disk holder (MAC disc holder; Amicon, MA). Stabilization and rinses were performed sequentially with 40 ml each of the solutions described above. Approximately 8.5 mg of IgG was bound (89% binding efficiency).

Purification of ES Antigens

Stage 24 chicken embryos were rinsed three times in ice-cold EBSS. Forty embryos (for large scale purification) or five embryos (for pilot purification) were homogenized (seven strokes with a glass Dounce) in 4 volumes of 20 mM Tris, pH 7.4, containing 150 mM NaCl (TBS), 1 mM PMSF, 1 mM EDTA, 1 μM pepstatin A, and 1 μM leupeptin. The homogenate was centrifuged at 10,000*g* for 30 min and the resulting supernatant spun at 100,000*g* for 90 min. One volume of TBS containing 0.1% (v/v) Tween 20 was added to the resulting supernatant, which was then incubated for 30 min on a rotating wheel, and filtered through a 0.4 μm membrane (CAMEO IIS; Micron Separations, Inc., MA). One fourth of the total sample volume was passed ten times back and forth through an affinity disk that had been equilibrated with TBS containing 0.05% Tween 20 (TBS-T), and the disk sequentially washed with 60 ml each (15 ml each for pilot purification) of TBS-T, Tris high salt buffer (TBSS; 20 mM Tris, 500 mM NaCl, pH 7.4) containing 0.05% Tween 20 (TBSS-T) and TBSS. ES proteins bound to the affinity disk were eluted with 0.1 M glycine, pH 2.3 (3 ml fractions were collected and neutralized). The A_{280} containing fractions were pooled, concentrated, and exchanged into PBS by centrifugal ultrafiltration. All procedures were performed at 4°C. Protein purification was monitored by A_{280} (Hitachi U-2000 spectrophotometer) and the final protein concentrations determined using a bicinchoninic acid assay with bovine serum albumin (BSA) as the standard (Micro BCA; PIERCE). Unfractionated total proteins of embryo were prepared by sonification of stage 24 embryos for five bursts of 5 s each (Sonifier; Branson, CT) in TBS containing 1 mM PMSF, 1 mM EDTA, 1 μM pepstatin A, and 1 μM leupeptin.

Electrophoresis and Western Blotting

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [1970] with the exception that sample buffer contained 63 mM Tris-HCl (pH 6.8), 2% SDS, 6 M urea, and 60 mM dithiothreitol (DTT; Boehringer Mannheim). Gels were stained with either coomassie [Fairbanks et al., 1971] or silver [Morrissey, 1981]. For western blot analyses, proteins were electroblotted onto Immobilon-P (Millipore) in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, pH 10.4 (CAPS; Sigma Chemical Co., St. Louis, MO) containing 6% methanol. The membranes were treated with TBS (pH 9.0) containing 5% BSA (Sigma Chemical Co., St. Louis, MO) for 1 h at 37°C to block nonspecific binding sites, rinsed in TBSS-T, reacted with primary antibodies diluted to 5 μg/ml in 1% BSA-TBSS-T for 1 h at room temperature, rinsed with TBSS-T, and incubated with alkaline phosphatase-conjugated secondary antibody (1:3,000 dilution, goat anti-rabbit IgG; BioRad) in 1% BSA-TBSS-T for 1 h at room temperature. Membranes were rinsed with TBSS-T and developed in alkaline phosphatase substrate solution (0.1 M carbonate buffer, pH 9.8, containing 1 mM $MgCl_2$, 0.15 mg/ml bromochloroindolyl phosphate and 0.3 mg/ml nitroblue tetrazolium).

For elution of proteins from gels, proteins in unfixed gels were identified by referring to a stained adjacent lane, minced with a homogenizer (Deltaware; Kimble, IL), and incubated

overnight in 2 vol of 50 mM ammonium bicarbonate (pH 7.8) containing 0.1% SDS at 37°C. Gel fragments were pelleted by centrifugation and the supernatant was exchanged into PBS and concentrated by centrifugal ultrafiltration (Ultrafree-MC; Millipore).

Microsequencing and Amino Acid Composition Analysis

Immunoaffinity purified ES proteins were subjected to SDS polyacrylamide gel electrophoresis (*200 μg* in two lanes; 10% total acrylamide gel pre-run for 40 min at 150 V). Proteins were electroblotted onto two layers of Immobilon P membrane in 10 mM CAPS buffer (pH 10.4) containing 10% methanol (4 h at 100 mA). The membranes were stained with 0.1% amido black (in 45% methanol/10% acetic acid) and destained with 90% methanol/2% acetic acid. Bands migrating side by side in duplicate lanes were cut out from both sheets and sequenced by the Medical College of Wisconsin Protein and Nucleic Acid Shared Facility on an Applied Biosystems Pulsed Liquid Phase sequencer (model 447A) with an on-line PTH amino acid analyzer. Sequence analysis was repeated twice to comfirm results. Total amino acid composition of each band was determined from acid hydrolysates of blotted proteins.

Enzyme-Linked Immunosorbent Assay (ELISA)

Microtiter plates (Falcon 3915; Becton Dickinson) were incubated overnight with serial dilutions of purified ES proteins or conalbumin (Type I, from chicken egg white, electrophoretic grade; Sigma Chemical Co., St. Louis, MO) in coating buffer (50 mM carbonate buffer, pH 9.6) at 4°C. After a brief rinse with TBS-T, plates were blocked with 1% BSA-TBS-T for 4 h at room temperature. Goat anti-rabbit transferrin (IgG fraction; Cappel) in 1% BSA-TBS-T was added at 1:200 dilution for 2 h at room temperature. Plates were washed five times with TBS-T. Alkaline phosphatase-conjugated secondary antibody (rabbit anti-goat IgG; Sigma Chemical Co., St. Louis, MO) was added at a 1:4,000 dilution, incubated for 2 h at room temperature, and washed as before. A solution of p-nitrophenylphosphate (Sigma Chemical Co., St. Louis, MO) at 1 mg/ml in 10 mM diethanolamine buffer (pH 9.6) containing 0.5 mM $MgCl_2$ was added, the plates incubated at room temperature, and the absorbance at 405 nm determined using a microplate reader (Model EL309, Bio-Tek Instruments).

Immunohistochemistry

Stage 13–20 embryos were cryoprotected by processing through a graded series of sucrose (10, 15, and 20%) in PBS, embedded in Tissue-Tek OCT Compound (Miles), and frozen in liquid nitrogen-cooled 2-methyl butane. Cryostat sections, 8 μm in thickness, were equilibrated in PBS for 10 min, and treated with blocking solution (1% BSA-PBS) for 1 h. The sections were reacted with ES antibodies or goat anti-rabbit transferrin IgG (Cappel) diluted 1:100 with 1% BSA-PBS for 1 h at room temperature. After rinsing in PBS, sections were incubated with a 1:100 dilution of fluorescene isothiocyanate-conjugated secondary antibody (rabbit anti-goat IgG; ICN Biomedicals) in 1% BSA-PBS for 1 h at room temperature. Sections were rinsed in PBS, mounted in 90% glycerol/10% PBS containing 0.2 M n-propylgallate [Giloh and Sedat, 1982], and examined with a Nikon Optiphot epifluorescent microscope. Primary antibody incubation was omitted or replaced with non-immune goat IgG at a relevant concentration for control sections.

RESULTS

As shown in Figure 1, proteins recognized by ES3 were localized to the extracellular matrix separating the endothelium from the myocardium. Staining was most intense in the extracellular matrix associated with the myocardium, similar to that reported previously for ES1 [Krug et al., 1987]. ES2 antibody primarily stained the extracellular matrix associated with transforming endothelial cells or their mesenchymal progeny [data not shown; Nakajima et al. in press]. Western blot analysis of unfractionated total proteins from stage 24 embryos showed that ES3 detected three proteins at 27, 44, and 63 kD and ES2 recognized mainly one protein at 44 kD (Fig. 2). Bands of similar mobility were detected by ES2 and ES3 antibodies on western blots of EDTA extract from stage 14–16 embryonic heart tissue (data not shown). Based on the similarity of immunostaining and western blot results to those reported previously for ES1 [Krug et al., 1987], ES3 was selected for subsequent immunopurification of ES antigens.

Owing to the observation that ES antigens are not restricted to the developing heart but are

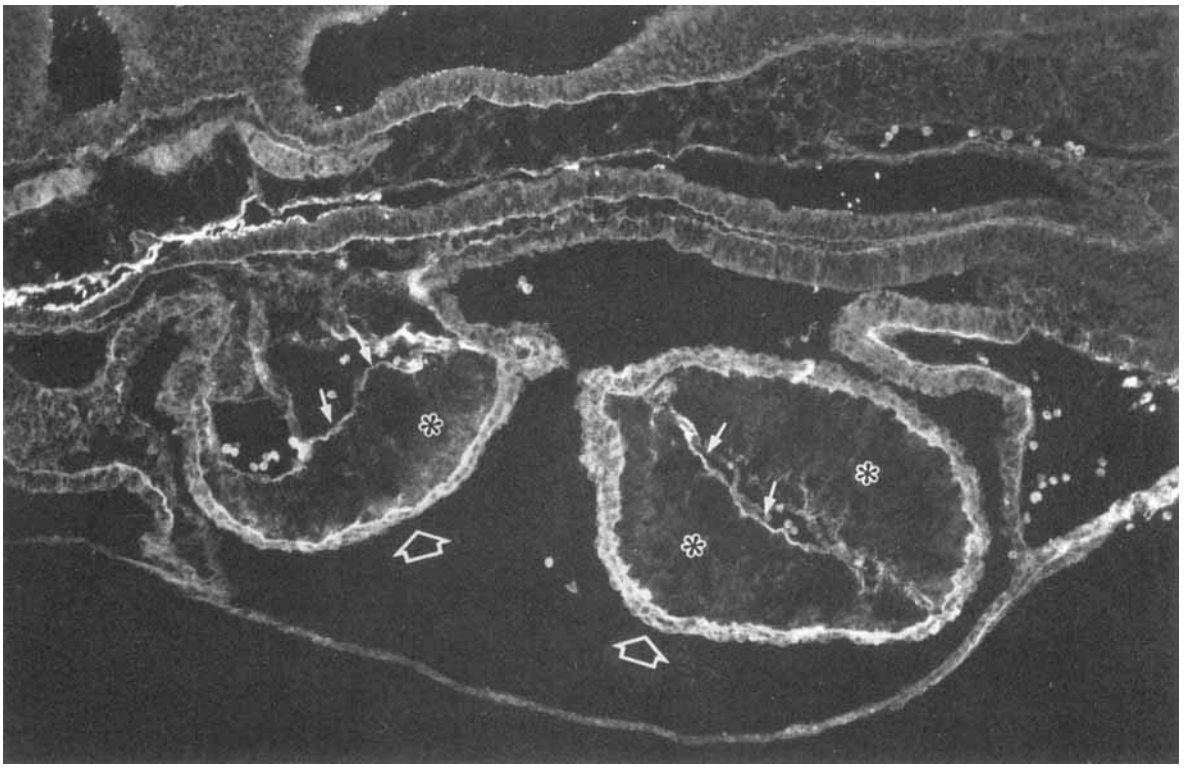

Fig. 1. Immunohistochemical localization of ES3 antigens in a stage 16 embryonic chick heart. ES3 antibodies recognized antigens in the extracellular matrix (asterisks), myocardium (large arrows), and at the basal surfaces of the endothelium (small arrows). Staining of extracellular matrix associated with the ventral foregut is also shown.

found at other sites of inductive interactions as well [Mjaatvedt et al., 1991; Isokawa et al., 1991], stage 24 whole embryos were used as a source for purifying ES antigens. Typically, 4 cycles of affinity purification with ES3 yielded 170 μg protein from the loading sample (100,000*g* supernatant fraction; 34 mg protein) which had been prepared from 40 stage 24 chicken embryos (80 mg protein). Thus, the ratio of purified protein to total starting material from stage 24 embryos is approximately 0.002 (500-fold enrichment). Purified preparations of ES3 antigens consisted of 27, 44, 63, and 70 kD proteins and a protein migrating at 51 kD (Fig. 3). The latter protein was shown by western analysis to be IgG leaching from the affinity support (Fig. 4).

To initially characterize ES3 antigens, samples of immunopurified protein were subjected sequentially to SDS-PAGE under nonreducing and reducing conditions. As shown in Figure 5, the mobility of all but the 27 kD polypeptide changed upon re-electrophoresis in the presence of DTT. The four ES3 protein bands migrating at 27, 44, 63, and 70 kD in the presence of DTT, migrated as 27, 41, 52, and 59 kD polypeptides, respectively, in the absence of DTT. Western blot analysis with ES3 IgG (Figs. 4, 6) showed that the 27 and 44 kD proteins were immunoreactive both with and without DTT, while the 63 kD protein was detectable only in the presence of DTT and the 70 kD protein was detectable only in the absence of DTT.

Amino-terminal microsequencing of the four ES proteins was attempted. Although the 27, 44, and 63 kD antigens all yielded heterogeneous sequence information, only the 70 kD antigen gave clean sequence data. The first ten amino acids determined (Ala-Pro-Pro-Lys-Ser-Val-Ile-Arg-Met-Asp) from the amino terminus of the 70 kD antigen were used to search a protein database (SWISS) by FASTA analysis with the University of Wisconsin Genetics Computer Group software. Results indicated that the first 8 amino acids were identical to those in chicken transferrin (conalbumin).

Three other biochemical analyses supported the identification of the 70 kD antigen as chicken transferrin. Total amino acid composition in

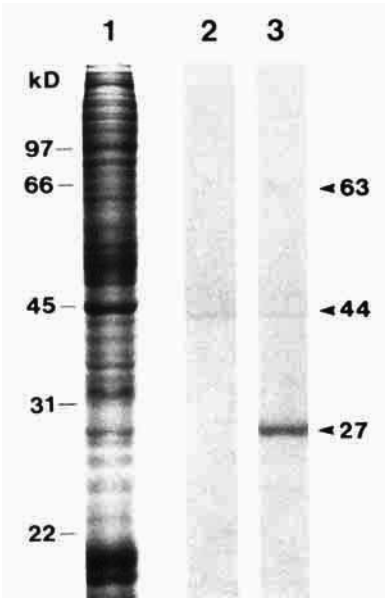

Fig. 2. Western blot analysis with ES2/ES3 polyclonal antibodies. Unfractionated total proteins (40 μg/lane) from stage 24 chicken embryos were separated on a 10% SDS-polyacrylamide gel and stained with Coomassie (lane 1). Proteins of duplicate lanes were electroblotted to Immobilon P membrane and stained with ES2 (lane 2) or ES3 (lane 3) antibody. Molecular weight markers are rabbit muscle phosphorylase b (97 kD), bovine serum albumin (66 kD), hen egg white ovalbumin (45 kD), bovine carbonic anhydrase (31 kD), and soybean trypsin inhibitor (22 kD).

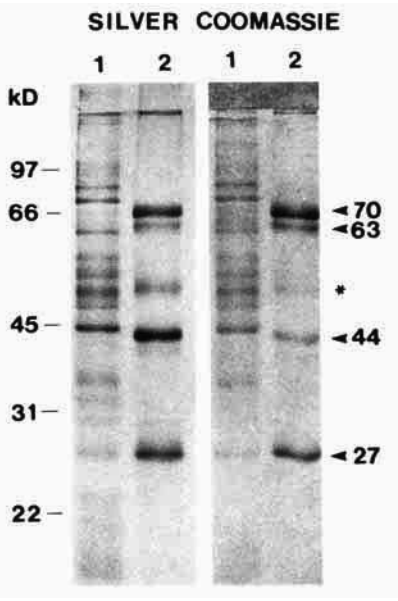

Fig. 3. Comparison of protein preparations loaded to and eluted from the ES3-affinity disk. The 100,000*g* supernatant fraction of a stage 24 embryo homogenate was subjected to ES3 immunoaffinity chromatography as described in Materials and Methods. Duplicate aliquots (1 μg/lane) of the supernatant fraction before chromatography (lane 1) and the bound/eluted material (lane 2) were solubilized under reducing conditions, separated on a 10% SDS-polyacryamide gel, and stained with either silver (left) or Coomassie (right). Four proteins migrating at 27, 44, 63, and 70 kD are enriched. The band indicated by the asterisk is shown to be a minor leaching of ES3 IgG from affinity disk (see Fig. 4, lane 4). Molecular weight markers are as in Figure 1.

acid hydrolysates of the 70 kD antigen was very similar to the deduced amino acid composition of chicken transferrin (conalbumin) except for a difference in glutamic acid composition (Table I). The mobility of the 70 kD antigen on SDS-PAGE as compared to a commercial preparation of chicken conalbumin was identical both in the presence and absence of DTT (Fig. 6). In addition, western blot analysis showed that chicken conalbumin was immunoreactive with ES3 only in the absence of DTT as was the 70 kD antigen (Fig. 6).

Using an antibody against rabbit transferrin, which was confirmed to cross-react with both chicken conalbumin and 70 kD ES antigen by ELISA assay (Fig. 7), immunohistochemistry was performed on cryostat sections of chick embryos. Anti-transferrin immunoreactivity was localized primarily to the heart, brain, neural tube, and somites, sites which previously were shown to also stain with ES1 antibodies [Mjaadvedt et al., 1991]. However, not all tissues that were positive for transferrin, e.g., liver and visceral yolk sac, were also stained by ES3 antibodies (data not shown). In the heart, before the onset of cushion tissue formation, the myocardium was intensely immunoreactive for transferrin while cardiac endothelium was negative (Fig. 8a). However, after the endothelium transforms into cushion mesenchyme, both the associated endothelium and migrating mesenchymal cells strongly expressed transferrin-immunoreactivity, particularly at cell surfaces, while expression in the myocardium remained high (Fig. 8b). Staining of the intervening extracellular matrix was not observed, either before or after the transformation of endothelial cells into mesenchyme. Control sections lacked specific fluorescence at any of these sites (not shown).

DISCUSSION

For this study, we prepared two polyclonal antibodies termed ES2 and ES3. Both were produced against an embryonic heart EDTA extract which was previously shown to have the poten-

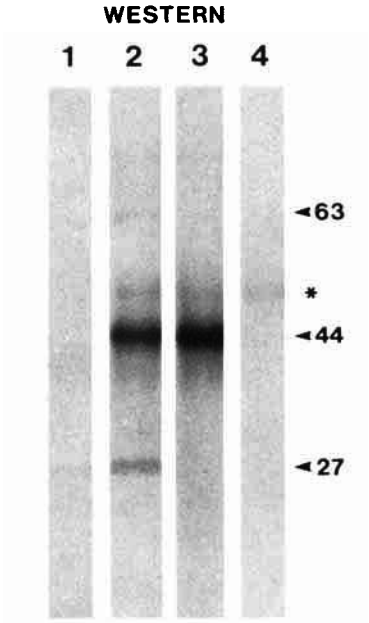

Fig. 4. Western blot analysis of protein preparations loaded to and eluted from ES3-affinity disk. One microgram/lane each of a loaded preparation (lane 1) and eluted preparation (lanes 2–4) were separated on a 10% SDS-polyacrylamide gel under reduced condition, blotted to Immobilon P membrane. Bands at 27, 44, and 63 kD were detected with ES3 antibodies, while the 70 kD protein in the eluted preparation was not immunoreactive with ES3 in samples treated with DTT (lane 2; see also Fig. 5). ES3 immunoaffinity purified proteins stained with ES2 antibodies (lane 3) showed that ES2 recognizes a 44 kD protein preferentially. By incubating a blot of ES3-purified proteins with secondary goat anti-rabbit antibody alone (lane 4), a band at 51 kD (asterisk) was shown to be rabbit IgG leached from the ES3-affinity support.

tial to induce cardiac endothelial-mesenchymal transformation in vitro [Krug et al., 1985, 1987; Mjaatvedt et al., 1989]. A similarly prepared, predecessor antibody, ES1, was able to completely remove the inductive potential of heart EDTA extracts indicating that ES antigens may directly or indirectly play a role in transforming heart endothelial cells into mesenchymal progeny. Culture bioassays showed that both ES2 and ES3 antibodies blocked AV endothelial transformation into mesenchyme [Nakajima et al., in press]. One of the antibodies, ES3, stained embryonic hearts and other tissue sites in a manner identical to ES1 and recognized similar antigens as ES1 in heart EDTA extracts. Because of the similarities between ES1 and ES3, we used this antibody to attempt to immunopurify ES3 antigens on a scale large enough to attempt N-terminal microsequencing.

From a total homogenate of stage 24 chick embryos, we were able to affinity purify four bands using ES3 antibodies and gel electrophoresis. However, for the three lower molecular weight bands the microsequencing approach yielded only vague findings possibly owing to more than one protein being present within each band or too little protein. We are presently attempting to identify these ES antigens by screening a stage 14–16 heart cDNA expression library [Rezaee et al., 1993].

For the 70 kD band, the sequence of the N-terminal eight amino acids matched exactly with those of chicken transferrin, while the amino acid composition for the 70 kD protein showed high-similarity to the deduced amino acid composition from chicken transferrin cDNA [Jeltsch et al., 1982]. ES3 antibody recognized both 70/59 kD protein and conalbumin on western blots under nonreduced conditions; similarly both these two proteins were also recognized by anti-rabbit transferrin antibody. In addition, the 70 kD protein co-migrated with conalbumin on SDS-PAGE under both reduced and nonreduced conditions. Therefore, the purified 70/59 kD ES protein is either transferrin or a similar sized transferrin-related molecule [Markelonis et al., 1982; Li et al., 1991].

Chicken transferrin is a β1-globulin in serum and its main producers are visceral yolk sac in the early embryo and liver in later stages [Adamson, 1982; Meek et al., 1985]. During development, however, several tissues and organs synthesize and store the protein including muscle, brain, skin, heart, lung, spinal cord, kidney, thymus, spleen, pancreas, salivary gland, and peripheral nerves [Meek et al., 1985; Skinner et al., 1991]. Conalbumin is a major egg white protein produced exclusively in the oviduct [Thibodeau et al., 1978]. Although gene expression for transferrin and conalbumin are regulated in a tissue-specific manner [Lee et al., 1978; Guillou et al., 1991] and their carbohydrate moieties are heterogeneous [Graham et al., 1975], they are products of the same gene [Graham et al., 1975; Thibodeau et al., 1978; Lee et al., 1978]. Because the 70 kD protein in the present study was purified from a total embryo homogenate, this protein could be derived from multiple tissues in the embryo. It is unlikely the 70 kD protein identified in this study by ES3 immunopurification was derived from the egg white as

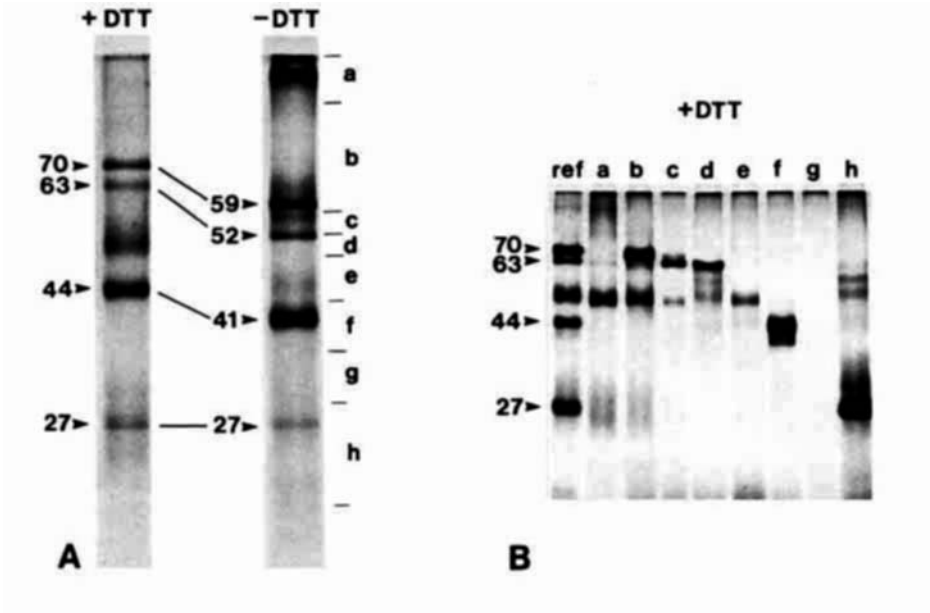

Fig. 5. Analysis of ES3-purified proteins by SDS-polyacrylamide gel electrophoresis in the presence and absence of DTT. Changes in mobility of individual ES proteins between reducing and nonreducing conditions are indicated in **A;** i.e., bands detected at 27, 44, 63, and 70 kD in the presence of DTT migrated at 27, 41, 52, and 59 kD, respectively, prior to treatment with reducing agent. IgG leached from the ES3 affinity matrix (51 kD band in reduced condition) moved to high molecular weight in the absence of reducing agent. These shifts in mobility were determined as follows: ES3-purified proteins were first separated under non-reducing conditions and each lane of the gel cut into eight pieces (a–h in the right lane of A). Proteins eluted from individual pieces were resolubilized in the presence of DTT, re-loaded to another 10% gel under reducing condition (a–h in **B**), and the mobility of the re-electrophoresed ES proteins compared to ES proteins treated similarly, but without prior electrophoresis ("ref" in B).

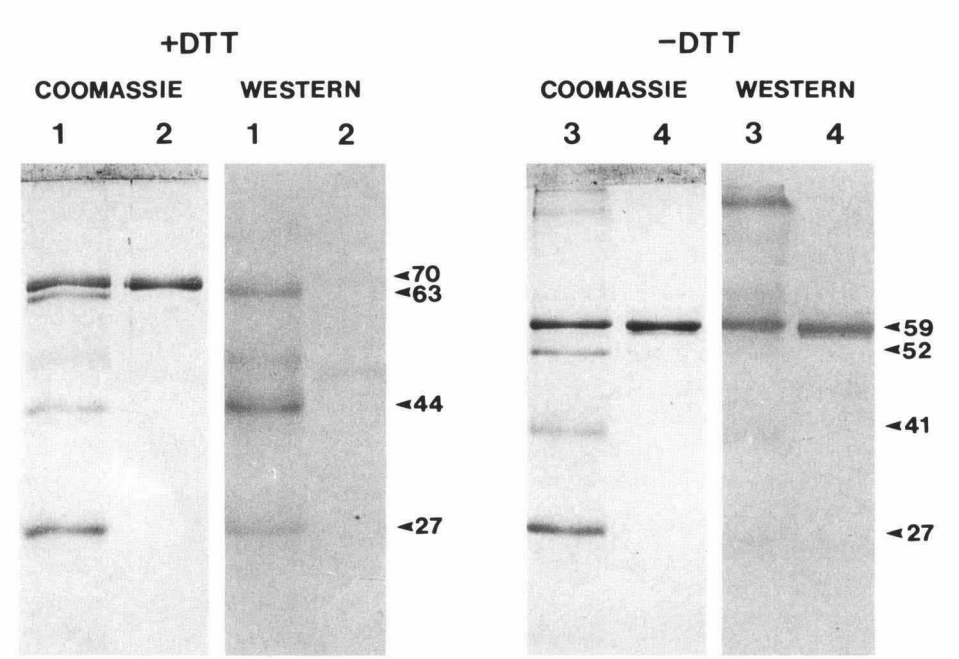

Fig. 6. Co-migration of the 70 kD ES3 antigen and chicken conalbumin upon SDS-polyacrylamide gel electrophoresis. ES proteins (2.5 μg/lane) under reducing (+DTT, lane 1) and nonreducing conditions (−DTT, lane 3) and chicken conalbumin (1 μg/lane) reduced (+DTT, lane 2) and nonreduced (−DTT, lane 4) were separated through a 10% polyacrylamide gel and stained with Commassie. Duplicate lanes were electrotransferred to Immobilon P membrane and stained with ES3 antibodies.

TABLE I. Comparison of Amino Acid Compositions between 70 kD ES Protein and Chicken Conalbumin*

	70 kD ES protein[a]	Conalbumin[b]
Lysine	8.4 (8.0)	8.5
Histidine	1.7 (1.6)	1.7
Arginine	3.8 (3.6)	4.2
Aspartic acid	10.5 (9.8)	11.5
Threonine	5.3 (5.0)	5.2
Serine	7.0 (6.5)	6.9
Glutamic acid	16.2 (15.2)	9.8
Proline	5.5 (5.1)	4.1
Glycine	9.3 (8.7)	7.6
Alanine	8.0 (7.6)	7.7
Cysteine	ND (4.4)	4.4
Valine	7.5 (7.1)	7.1
Methionine	1.4 (1.3)	1.6
Isoleucine	4.0 (3.8)	3.9
Leucine	8.5 (8.0)	7.1
Tyrosine	3.0 (2.9)	3.1
Phenylalanine	4.1 (3.8)	3.9
Tryptophan	ND (1.6)	1.6

*Results are expressed as numbers of residues per 100 amino acids.

[a]The values were calculated based on 18 amino acids surviving acid hydrolysis. The values in parenthesis were calculated assuming that the amount of cysteine and tryptophan in the 70 kD ES protein was the same as in conalbumin. Lysine values were corrected for disubstitution during the sequencing process.

[b]The values are calculated based on amino acid numbers deduced from conalbumin cDNA sequence.

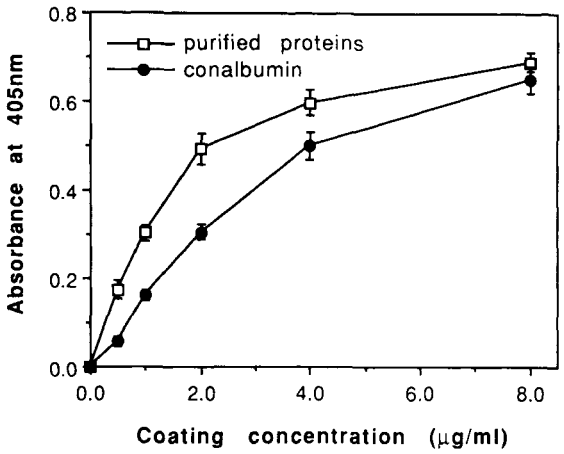

Fig. 7. Reactivity of goat anti-rabbit transferrin towards ES proteins and chicken conalbumin by ELISA analysis. Data are expressed as mean ± standard deviation (n = 6).

antigens used to prepare the antibody were obtained from EDTA extracts from over 2,000 isolated hearts that had been washed extensively. Rather, it is more likely that at least part of the 70 kD protein purified and identified in this study as transferrin was derived from embryonic hearts. When treated with anti-rabbit transferrin antibodies, which cross-reacted with the 70 kD protein and conalbumin, heart tissues were stained intensely, particularly the myocardium prior to the onset of endothelial transformation. Of course, either Northern or in situ hybridization analyses will be required to determine conclusively if transferrin immunostaining in the heart represents synthesis or uptake.

Although the function of transferrin as a nutritional factor has been well characterized [Saito et al., 1982; Landschulz et al., 1984; Hagiwara et al., 1987], the full role of transferrin during embryogenesis cannot be limited to that of a simple iron transport molecule [Tsunoi et al., 1984; Sanders, 1986; Kovar et al., 1989; Denstman et al., 1991]. For example, Ekbloom and Thesleff and their co-workers have evidence for transferrin being a mediator of inductive tissue interaction in kidney development [Ekblom et al., 1981, 1983; Thesleff et al., 1984; Landschulz et al., 1984]. Using an organ culture system, it was shown that 1) metanephric mesenchyme (the target tissue) acquired transferrin-responsiveness, or competence, as a consequence of induction by a yet unknown substance in ureter buds or spinal cord (the inductor tissue); 2) the target mesenchyme, the inductor, and transferrin all three needed to be present simultaneously for a certain time period in order for the mesenchyme to become competent; and 3) the competent metanephric mesenchyme responded to transferrin by proliferation and showed morphogenesis of tubule formation.

A similar induction mechanism related to transferrin has been reported in some established cell lines. In these systems, cells are activated to proliferate first by the action of "competence factors," such as platelet derived growth factor, tumor necrosis factor-α, transforming growth factor-β and phorbol ester. Once activated, competent cells progress into the S-(synthetic) phase of their cell cycle by the action of "progression factors," one of which has been identified as transferrin [Smith, 1981; Denstman et al., 1991]. Without the presence of transferrin, cells halt at the G1-phase and do not differentiate. The biological activity of transferrin in these systems was not related to its iron-

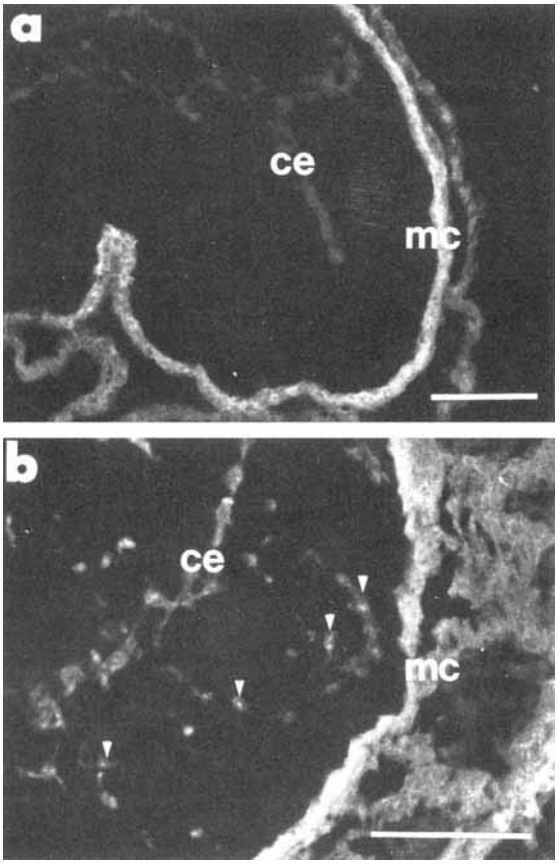

Fig. 8. Fluorescent immunohistochemistry with anti-rabbit transferrin. Unfixed cryostat sections of chicken embryonic hearts at stage 13 (**a**) and stage 19 (**b**) were stained with goat anti-rabbit transferrin which was shown to cross-react with chicken transferrin (see Fig. 7). At stage 13 (a) prior to the initiation of endothelial-mesenchymal transformation, the antibody stained myocardium (mc) intensely but not cardiac endothelium (ce). At stage 19 (b) after the initiation of the transformation, both cardiac endothelium (ce) and migrating mesenchymal cells (arrowheads) became positive, while immunoreactivity in the myocardium (mc) persisted. Bars, 100 μm.

carrying capacity [Smith, 1981; Denstman et al., 1991], underscoring its potential to function in developmental tissue interactions in ways other than merely nutritive.

In the present study, the immunological expression of transferrin in the developing heart was strikingly synchronous with the initiation of cardiac endothelial-mesenchymal transformation. Cardiac endothelium was completely negative with anti-transferrin *before* this transformation but positive *after* its transition into mesenchyme by signals secreted from the myocardium [Krug et al., 1985, 1987]: both the transforming endothelium and associated migrating mesenchymal cells strongly expressed transferrin. Whether this represented an upregulation of the transferrin gene or increased expression of transferrin receptors by the targeted endothelial cells remains to be determined. In either case, an autocrine role for this protein is suggested as little if any transferrin was immunologically detected in the extracellular matrix separating the myocardium from endothelium.

The identification of one ES antigen as transferrin and its localization on specific populations of endothelial cells potentially provides an important new insight into the process of how embryonic epithelial cells transform into mesenchyme. In this regard, a recent study which identified a lung-derived growth factor as transferrin might be comparable with our present study [Cavanaugh et al., 1991]. It was shown that the factor stimulated mitogenic activity in metastasizing tumor cells but not in nonmetastasizing tumor cells. Expression of transferrin would seem to be but one similarity recently found between transformation and migration in normal embryogenesis and the metastasizing process in tumorgenesis [Yamada, 1983; Klein et al., 1985; Van Roy et al., 1986; Harrison, 1989].

To understand the molecular events of inductive tissue interactions such as occurs between the myocardium and endothelium will require identification of those regulatory signals that operate in a paracrine manner. Growth factors have been implicated as such paracrine mediators; however, the distribution of ES antigens at several sites of inductive tissue interactions also makes them candidates for consideration. Although we did not succeed in molecular characterization of the other three ES antigens, the molecular weights identified suggest they are larger than characteristic growth factors. Thus, our ability to purify these antigens may be an important first step in understanding genetic regulation of pattern formation during embryogenesis.

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REFERENCES

Adamson ED (1982): The location and synthesis of transferrin in mouse embryos and teratocarcinoma cells. Dev Biol 91:227–234.

Akhurst RJ, Lehnert SA, Faissner A, Duffie E (1990): TGF, beta in murine morphogenetic processes: The early embryo and cardiogenesis. Development 108:645–656.

Cavanaugh PG, Nicolson GL (1991): Lung-derived growth factor that stimulates the growth of lung-metastasizing tumor cells. Identification as transferrin. J Cell Biochem 47:261–271.

Clark EB, Takao A (1990): Overview: A focus for research in cardiovascular development. In Clark EB, Takao A (eds): "Developmental Cardiology: Morphogenesis and Function." Mount Kisco, NY: Futura Publishing, pp 3–12.

Crossin KL, Hoffman S (1991): Expression of adhesion molecules during the formation and differentiation of the avian endocardial cushion tissue. Dev Biol 145:277–286.

Denstman S, Hromchak R, Guan XP, Bloch A (1991): Identification of transferrin as a progression factor for ML-1 human myeloblastic leukemia cell differentiation. J Biol Chem 266:14873–14876.

Ekblom P, Lehtonen E, Saxtn L, Timpl R (1981): Shift in collagen type as an early response to induction of the metanephric mesenchyme. J Cell Biol 89:276–283.

Ekblom P, Thesleff I, Saxtn L, Miettinen A, Timpl R (1983): Transferrin as a fetal growth factor: Acquisition of responsiveness related to embryonic induction. Proc Natl Acad Sci USA 80:2651–2655.

Fairbanks G, Steck TL, Wallach DFH (1971): Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 10:2606–2617.

Funderberg FM, Markwald RR (1986): Conditioning of native substrates by chondroitin sulfate proteoglycans during cardiac mesenchymal cell migration. J Cell Biol 103: 2475–2487.

Giloh H, Sedat JW (1982): Fluorescence microscopy. Reduced photobleaching of rhodamine and fluorescein protein complexes by n-propylgallate. Science 217:1252–1255.

Graham I, Williams J (1975): A comparison of glycopeptides from the transferrins of several species. Biochem J 145: 263–279.

Guillou F, Zakin MM, Part D, Boissier F, Schaeffer E (1991): Sertoli cell-specific expression of the human transferrin gene. J Biol Chem 266:9876–9884.

Hagiwara Y, Saito K, Atsumi S, Ozawa E (1987): Iron supports myogenic cell differentiation to the same degree as does iron-bound transferrin. Dev Biol 120:236–244.

Hamburger V, Hamilton HL (1951): A series of normal stages in the development of the chick embryo. J Morphol 38:49–92.

Harrison F (1989): The extracellular matrix and cell surface, mediators of cell interactions in chicken gastrulation. Int J Devl Biol 33:417–438.

Isokawa K, Krug EL, Markwald RR (1991): Identification of ectodermal extracellular matrix proteins that inhibit chondrogenesis of early limb mesoderm. J Cell Biol 115:447a.

Jeltsch JM, Chambon P (1982): The complete nucleotide sequence of the chicken ovotransferrin mRNA. Eur J Biochem 122:291–295.

Jessell TM, Melton DA (1992): Diffusible factors in vertebrate embryonic induction. Cell 68:257–270.

Klein G, Klein E (1985): Evolution of tumours and the impact of molecular oncology. Nature 315:190–195.

Kovar J, Franek F (1989): Growth-stimulating effect of transferrin on a hybridoma cell line: Relation to transferrin iron-transporting function. Exp Cell Res 182:358–369.

Krug EL, Runyan RB, Markwald RR (1985): Protein extracts from early embryonic hearts initiate cardiac endothelial cytodifferentiation. Dev Biol 112:414–426.

Krug EL, Mjaatvedt CH, Markwald RR (1987): Extracellular matrix from embryonic myocardium elicits an early morphogenetic event in cardiac endothelial differentiation. Dev Biol 120:348–355.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Landschulz W, Thesleff I, Ekblom P (1984): A lipophilic iron chelator can replace transferrin as a stimulator of cell proliferation and differentiation. J Cell Biol 98:596–601.

Lee DC, McKnight GS, Palmiter RD (1978): The action of estrogen and progesterone on the expression of the transferrin gene. J Biol Chem 253:3494–3503.

Li Y, Moczydlowski E (1991): Purification and partial sequencing of Saxiphilin, a Saxitoxin-binding protein from the Bullfrog, reveals homology to transferren. J Biol Chem 266:15481–15487.

Markelonis GJ, Bradshaw RA, Oh TH, Johnson JL, Bates OJ (1982): Sciatin is a transferrin-like polypeptide. J Neurochem 39:315–320.

Meek J, Adamson ED (1985): Transferrin in foetal and adult mouse tissues. Synthesis, storage and secretion. J Embryol Exp Morphol 86:205–218.

Mjaatvedt CH, Krug EL, Markwald RR (1991): An antiserum (ES1) against a particulate form of extracellular matrix blocks the transition of cardiac endothelium into mesenchyme in culture. Dev Biol 145:219–230.

Mjaatvedt CH, Markwald RR (1989): Induction of an epithelial-mesenchymal transition by an in vivo adheron-like complex. Dev Biol 136:118–128.

Morrissey JH (1981): Silver stain for proteins in polyacrylamide gels: A modified procedure with enhanced uniform sensitivity. Anal Biochem 117:307–310.

Nakajima Y, Krug EL, Markwald RR (in press): Myocardial regulation of transforming growth factor beta expression by outflow tranct endothelium in the early embryonic chick heart. Dev Biol.

Rezaee M, Isokawa K, Halligan N, Markwald RR, Krug EL (1993): Identification of an extracellular 130-kDa protein involved in early cardiac morphogenesis. J Biol Chem 268:14404–14411.

Robert B, Sassoon D, Jacq B, Gehring W, Buckingham M (1989): Hox-7, a mouse homeobox gene with a novel pattern of expression during embryonic heart development. EMBO J 8:91–100.

Saito K, Hagiwara Y, Hasegawa T, Ozawa E (1982): Indispensability of iron for the growth of cultured chick cells. Dev Growth Diff 24:571–580.

Sanders EJ (1986): Changes in the transferrin requirement of cultured chick embryo mesoderm cells during early differentiation. J Embryol Exp Morphol 95:81–93.

Skinner SJM, Somervell CE (1991): Transferrin gene expression and transferrin immunolocalization in developing foetal rat lung. J Cell Sci 99:651–656.

Smith JC (1981): Growth factors and pattern formation. J Embryol Exp Morphol 65:187–207.

Thesleff I, Ekblom P (1984): Role of transferrin in branching morphogenesis, growth and differentiation of the embryonic kidney. J Embryol Exp Morphol 82:147–161.

Thibodeau SN, Lee DC, Palmiter RD (1978): Identifical precursors for serum transferrin and egg white conalbumin. J Biol Chem 253:3771–3774.

Tsunoi M, Hakeda Y, Kurihara N, Maeda N, Utsumi N, Kumegawa M (1984): Effect of transferrin on alkaline phophatase activity and collagen synthesis in osteoblastic cells derived from newborn mouse calvaria. Exp Cell Res 153:240–244.

Yamada KM (1983): Cell surface interactions with extracellular material. Annu Rev Biochem 52:761–799.

Van Roy FM, Messiaen L, Liebaut J, Gao CH, Dragonetti CH, Fiers WC, Mareel MM (1986): Invasiveness and metastatic capability of rat fibroblast-line cells before and after transfection with immortalizing and transforming genes. Cancer Res 46:4787–4795.