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Competency of embryonic cardiomyocytes to undergo Purkinje fiber differentiation is regulated by endothelin receptor expression

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SUMMARY

Purkinje fibers of the cardiac conduction system differentiate from heart muscle cells during embryogenesis. In the avian heart, Purkinje fiber differentiation takes place along the endocardium and coronary arteries. To date, only the vascular cytokine endothelin (ET) has been demonstrated to induce embryonic cardiomyocytes to differentiate into Purkinje fibers. This ET-induced Purkinje fiber differentiation is mediated by binding of ET to its transmembrane receptors that are expressed by myocytes. Expression of ET converting enzyme 1, which produces a biologically active ET ligand, begins in cardiac endothelia, both arterial and endocardial, at initiation of conduction cell differentiation and continues throughout heart development. Yet, the ability of cardiomyocytes to convert their phenotype in response to ET declines as embryos mature. Therefore, the loss of responsiveness to the inductive signal appears not to be associated with the level of ET ligand in the heart. This study examines the role of ET receptors in this age-dependent loss of inductive responsiveness and the expression profiles of three different types of ET receptors, ET_A, ET_B and ET_{B2}, in the embryonic chick heart. Whole-mount in situ hybridization analyses revealed that ET_A was ubiquitously expressed in both ventricular and atrial myocardium during heart development, while ET_B was predominantly expressed in the atrium and the left ventricle. ET_{B2} expression was detected in valve leaflets but not in the myocardium. RNase protection assays showed that ventricular expression of ET_A and ET_B increased until Purkinje fiber differentiation began. Importantly, the levels of both receptor isotypes decreased after this time. Retrovirus-mediated overexpression of ET_A in ventricular myocytes in which endogenous ET receptors had been downregulated, enhanced their responsiveness to ET, allowing them to differentiate into conduction cells. These results suggest that the developmentally regulated expression of ET receptors plays a crucial role in determining the competency of ventricular myocytes to respond to inductive ET signaling in the chick embryo.

Key words: Cardiac conduction system, Purkinje fibers, Endothelin receptor, Retrovirus, Embryonic chicken heart, Myocardium

INTRODUCTION

A rhythmic heart beat in higher vertebrates is coordinated by the pacemaking action potentials of the cardiac conduction system (Tawara, 1906; Bozler, 1942). This rhythmic sequence of activation initiates at the sinoatrial node and is conducted as an action potential across the atrial chambers to the atrioventricular node (Tawara, 1906). The action potential is then propagated along the atrioventricular bundle and its branched limbs, finally spreading into the ventricular muscle via the Purkinje fiber network (Purkinje, 1845; His, 1893; Kolliker, 1902; Tawara, 1906). In the avian heart, Purkinje fibers are identified as a tissue network ramifying along the endocardium and branching coronary vascular bed (Vassal-Adams, 1982; Gourdie et al., 1995). Purkinje fibers are distinguished from ordinary myocytes by their unique cellular and molecular characteristics (Sartore et al., 1987; Mikawa and Fischman, 1996; Schiaffino, 1997; Moorman et al., 1998; Takebayashi-Suzuki et al., 2001).

Retroviral cell lineage studies in the chicken embryonic heart (Gourdie et al., 1995; Cheng et al., 1999) have demonstrated that a subset of contractile myocytes undergo trans-differentiation into conduction cells, and that this conversion from contractile to conducting phenotype in the myocyte lineage invariably and restrictively occurs adjacent to the endocardium and developing coronary arteries. A recent study of the *En2/lacZ* mouse heart suggests that murine Purkinje fibers differentiate exclusively along the endocardium (Rentschler et al., 2001). Unique sites of Purkinje fiber recruitment have led to the hypothesis that conduction cells may be induced by receiving paracrine signals originating from endocardial and arterial vascular tissues (Mikawa and Fischman, 1996; Mikawa, 1998a; Mikawa, 1998b).

Consistent with this idea, instructive cue(s) from cardiac endothelium have been shown to be necessary and sufficient for recruiting contractile myocytes to form Purkinje fibers in the embryonic chick heart (Hyer et al., 1999; Takebayashi-Suzuki et al., 2000). Furthermore, it has been demonstrated that

beating embryonic myocytes are competent to respond to endothelin (ET), a paracrine factor secreted by endothelial cells (Yanagisawa et al., 1988; Masaki et al., 1991), and can be prompted by this factor to differentiate into Purkinje fibers both in vivo (Takebayashi-Suzuki et al., 2000) and in vitro (Gourdie et al., 1998).

In contrast to embryonic cardiomyocytes, mature heart muscle cells respond to ET by undergoing hypertrophic growth (Ito et al., 1991), suggesting that responsiveness of myocytes to ET may be a distinct, developmentally regulated, process. Indeed, the frequency of ET-dependent conversion of myocytes from a contractile to a conductive Purkinje phenotype declines as embryonic development proceeds (Gourdie et al., 1998). ET is post-translationally processed from its precursor, preproET (Xu et al., 1994). PreproET is first cleaved by furin proteases into a biologically inactive intermediate, bigET, which is further processed with the highly specific ET-converting enzyme (ECE1) into biologically active ET (Xu et al., 1994; Emoto and Yanagisawa, 1995). ET signaling is triggered by binding of ET to its G protein-coupled receptors, ET_A, ET_B and ET_{B2} (Arai et al., 1990; Sakurai et al., 1990; Nataf et al., 1996). Although ligand-receptor interactions are well characterized, the mechanism underlying developmental changes in competency of myocytes to respond to ET signaling remains uncertain.

The detailed expression pattern of ET-related genes in the embryonic heart has only been established for the tubular stage heart (Kurihara et al., 1995; Clouthier et al., 1998; Yanagisawa et al., 1998). ET1 expression at this stage is restricted to the outflow tract endocardium and endocardial cushions (Kurihara et al., 1995; Clouthier et al., 1998), while ET_A is expressed by all myocytes and is absent from the endocardium (Clouthier et al., 1998; Yanagisawa et al., 1998). By contrast, ECE1 expression is present in the endocardium and absent from myocytes at this developmental stage (Yanagisawa et al., 1998). Thus, each of these components is expressed by different cell types at distinct locations within the tubular stage heart. However, little is known about the expression profile of ET-related genes during conduction system development, which occurs later in embryogenesis.

Our previous studies of the embryonic chick heart have shown that expression of ECE1, a key enzyme for active ET production, begins predominantly in endocardial and coronary arterial endothelia, just prior to initiation of juxtaposed Purkinje fiber differentiation (Takebayashi-Suzuki et al., 2000). Importantly, ECE-1 expression in these cardiac endothelia continues throughout heart development and becomes more robust as Purkinje fiber differentiation proceeds (Takebayashi-Suzuki et al., 2000). It is therefore unlikely that the loss of responsiveness to the inductive signal is associated with the level of ECE1 that produces ET ligands in the heart.

In the present study, we have examined the potential roles of ET receptors in the regulation of myocyte conversion to Purkinje fibers in response to ET signaling. Evidence is presented demonstrating that expression of ET receptors in the heart is developmentally regulated, consistent with a possible role in Purkinje fiber differentiation. We show that induced upregulation of ET_A expression in ventricular myocytes gives rise to enhanced and/or prolonged responsiveness to added ET ligand. The data presented suggest a close association between the level of ET-receptor expression and the developmentally regulated ability of myocytes to be converted to impulse-conducting cells.

MATERIALS AND METHODS

Cloning of chicken ET-receptor cDNAs

Partial cDNAs of chicken ET_A (Kempf et al., 1998) and quail ET_B (Lecoin et al., 1998) were labeled with ^{32}P by random priming and used to screen an embryonic day 7 chicken cDNA library (Takebayashi-Suzuki et al., 2000). Approximately $2\text{-}3\times10^5$ phages plaques were screened for cloning of ET_A, ET_B and ET_{B2} cDNAs. Seven ET_A and five ET_B/ET_{B2} phage clones were isolated. The longest clone of each cDNA type was selected with Southern blot and restriction enzyme analyses, and subcloned into the *Not*I site of pBluescript II SK (+) (Stratagene). The chicken ET_A, ET_B and ET_{B2} cDNAs were sequenced in both directions, using the dideoxynucleotide sequencing technique, and the sequence data have been lodged with the EMBL/GenBank/DDBJ data base (BankIt449603 AF472616, BankIt449605 AF472617, and BankIt442634 AF472618, respectively).

In situ hybridization

The *Eco*RI/*Spe*I (316 bp) fragment of the ET_A clone, *Hin*dIII/*Not*I (741 bp) fragment from the ET_B clone (designated cET_B741HN) and *Bam*HI/*Hin*dIII (414 bp) fragment from the ET_{B2} clone were subcloned into pBluescript II vector and used as templates for riboprobe synthesis. The *Spe*I/*Eco*RI fragment (632 bp) of a chicken ventricular myosin heavy chain cDNA, pVMHC1 (Bisaha and Bader, 1991), was ligated into pBluescript II. Digoxigenin-conjugated riboprobes were generated, using standard riboprobe protocols (Boehringer Mannheim). Whole-mount in situ hybridization was carried out as described elsewhere (Takebayashi-Suzuki et al., 2000).

RNase protection assay

*Xba*I/*Eco*RI fragment (210 bp) containing a 3′-flanking region of chicken ET_A cDNA was subcloned into pBluescript II, linearized with *Xba*I and transcribed with T7 RNA polymerase, yielding antisense probes of 273 bases to protect 210 bases of ET_A mRNA. The cET_B741HN was linearized with *Mfe*I and transcribed with T3 RNA polymerase, generating antisense probes of 165 bases to protect 136 bases of ET_B mRNA. The pVMHC1 was linearized with *Bam*HI and transcribed with T7 RNA polymerase, producing antisense probes of 317 bases to protect 251 bases of VMHC1 mRNA. A partial cDNA of chicken GAPDH (Panabieres, 1984) was cloned from mRNA of chicken day 6 embryos using RT-PCR with primers 5′-ACGC-CATCACTATCTTCCAG-3′ (forward) and 5′-CAGCCTTCACTAC-CCTCTTG-3′ (reverse). The PCR product was subcloned into pCRII TA-cloning vector (Invitrogen) and transcribed with T7 RNA polymerase, producing antisense probes to protect 114bp of GAPDH mRNA. The RNase protection assay was performed according to the manufacturer's protocol using an RPAIII kit (Ambion). The specific activity of all probes was adjusted to 1×10^6 cpm/ng, except for 1×10^4 cpm/ng of VMHC1 probe. Analysis of protected fragments was performed as described (Alyonycheva et al., 1997).

Reverse transcriptase-polymerase chain reaction (RT-PCR) assay

Total RNA was extracted with Trizol Reagent from E3 heart tubes and those cultured for 1, 2, 3 and 10 days. Using random hexamers, cDNAs were synthesized from 1.5 μg of total RNA with AMV reverse transcriptase (Roche). PCR was carried out as described by Roche cDNA synthesis kit for RT-PCR. ET_A cDNA was amplified with primers 5′-CCATTGGTTTTGCCGTGG-3′ (forward) and 5′-TCCT-GATTGATTCTGAAGTAGTGTC-3′ (reverse), while ET_B cDNA was synthesized with primers 5′-AAGTACCAGGCAGAACACAG-3′

(forward) and 5′-CATCCCCATCAGCGTCTACA-3 (reverse). GAPDH cDNA was amplified as described (Schultheiss et al., 1995) with primers 5′-ACGCCATCACTATCTTCCAG-3′ (forward) and 5′-CAGCCTTCACTACCCTCTTG-3′ (reverse). All primers were annealed at 55°C. ET_A and ET_B PCR products (5 μl) and 1 μl of GAPDH PCR products were applied, respectively, to agarose gels and stained with Ethidium Bromide. Gels were analyzed as described above.

Northern hybridization

Northern blot analysis was performed according to standard procedures. In brief, 20 μg of total RNA was resolved by electrophoresis and transferred onto Hybond-XL membranes (Amersham). A coding region of ET_A cDNA was ^{32}P-labelled with a random priming labeling kit and used to probe blots at 65°C in Rapid-Hyb buffer (Amersham).

ET_A virus

A prospective poly-adenylation signal in ET_A cDNA was eliminated with PCR using degenerate primers 5′-CCATTGGTTTGCCGTGG-3′ (forward) and 5′-TCCTGATTGATTCTGAAGTAGTGTC-3′ (reverse). Amplified fragments were ligated into pCRII TA-cloning vector and fused with the *Hin*dIII/*Kpn*I fragment of ET_A encoding the 5′ UTR and 5′ half of protein coding region. The ET_A cDNA was ligated into the *Xba*I site upstream of an internal ribosome entry sequence fused to *lacZ* of the spleen necrosis virus-based replication defective retroviral vector, pCXIZ (Mikawa, 1995; Mima et al., 1995). The resulting plasmid was designated pCXIZ-ETA. Propagation of CXIZ-ETA virus and control CXL virus encoding only *lacZ* (Mikawa et al., 1991) was carried out as described (Mikawa et al., 1996). The ventricular myocardium was infected in ovo with virus as described (Mikawa et al., 1992).

Tissue culture and immunohistochemistry

Heart tubes or ventricular segments were dissected 24 hours after viral infection and cultured for various days in DMEM containing 10% FBS, 1% chick embryonic extract, in the presence or absence of ET1 peptide, and processed for whole tissue or section immunostaining, as described (Takebayashi-Suzuki et al., 2000). Primary antibodies, ALD58 against a Purkinje fiber marker, slow skeletal muscle myosin heavy chain (sMyHC) (Gonzalez-Sanchez and Bader, 1985), MF20 against sarcomeric MyHCs and anti-β-Gal (5 prime 3 prime Company), were used in the present study and detected with secondary antibodies Alexa-488-conjugated goat anti-mouse and Alexa-594-conjugated goat anti-rabbit (Molecular Probes) at a dilution of 1:200. All images of heart tubes and frozen sections were captured directly with a digital camera.

RESULTS

Chicken ET receptor cDNAs

To examine the expression pattern and function of ET receptors in the embryonic chick heart during Purkinje fiber differentiation, cDNAs of chicken ET_A, ET_B and ET_{B2} were cloned. A 2988bp clone (AF5-3) encoded a 1263bp open reading frame flanked by 5′ and 3′ untranslated regions (Fig. 1A). The deduced amino acid sequence of the AF5-3 clone consisted of 421 amino acid residues identical to the reported ET_A sequence (Kempf et al., 1998). Therefore, the AF5-3 clone was designated a full-length cDNA of chicken ET_A. A 1564bp clone (B2) encoded a 1068bp open reading frame with a 3′ noncoding region, lacking 5′ noncoding region and the translation initiation site (Fig. 1B). The deduced amino acid sequence of the B2 clone revealed 356 amino acid residues

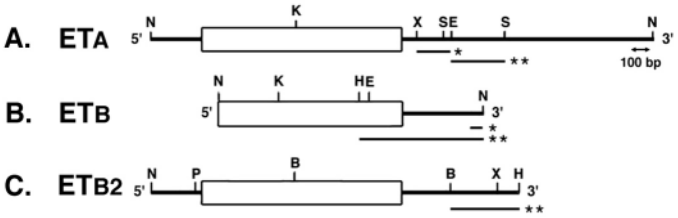

Fig. 1. Chicken ET_A, ET_B and ET_{B2} cDNA clones. Asterisks and double asterisks represent regions used for RNase protection assay and in situ hybridization, respectively. Open box, protein-coding region; line, non-coding region; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Spe*I; X, *Xba*I.

with homology of 99.4% with quail ET_B, 73% with quail ET_{B2} (Lecoin et al., 1998) and 65.5% with chick ET_A. Based on these data, the B2 clone was designated a partial cDNA of chick ET_B. A 2225 bp clone (B5) encoded a 1308 open reading frame flanked with 5′ and 3′ noncoding regions (Fig. 1C). The 436 amino acid residues deduced from the B5 clone showed the sequence homology of 97.5% with quail ET_{B2} (Lecoin et al., 1998), 65.3% with chick ET_A and 72.3% with chick ET_B. Therefore, the B5 clone was designated a full-length chick ET_{B2} cDNA. A database search and comparison revealed that chick ET_A, ET_B and ET_{B2} maintain all of the structural characteristics of the G protein-coupled ET-receptor family with seven membrane domains (data not shown). Less than 50% homology of nucleotide sequences was detected in 3′ non-coding regions among ET-receptor cDNAs. The unique 3′ non-coding regions (Fig. 1) were used in the following whole-mount in situ hybridization and RNase protection analyses.

Differential expression of ET receptor isotypes in the embryonic heart

Expression of three ET receptor isotypes in embryonic hearts was examined by whole-mount in situ hybridization prior to and during Purkinje fiber differentiation, which becomes detectable at embryonic days (E) 10-13 (Gourdie et al., 1995; Takebayashi-Suzuki et al., 2000). Probing for positive expression of *VMHC1*, a ventricular muscle-specific myosin heavy chain, ventricular myocytes from which Purkinje fibers differentiate were first identified and distinguished from other cardiac cell types (Fig. 2A-D). At E3, just after the onset of heart beat, robust hybridization of *VMHC1* was already evident in the heart tube (Fig. 2A) and became exclusive to the ventricular myocardium in later development (Fig. 2B-D), as described previously (Yutzey et al., 1995).

ET_A transcripts were detected in both ventricular and atrial myocardial walls throughout heart development (Fig. 2E-I). At E3, the heart tube exhibited *ET_A* signals that were only slightly above background levels (Fig. 2E), while robust signals were seen in proximal regions of branchial arches 1, 2 and 3. By E6, hybridization signals in the heart became more apparent ubiquitously in both the atrial and ventricular myocardium (Fig. 2F) and continued until E10 (Fig. 2G), when overt Purkinje fiber differentiation begins. Importantly, *ET_A* signals in the myocardium gradually declined as embryos matured (Fig. 2H,I), although the ubiquitous expression pattern was still evident as seen in our previous studies (Takebayashi-Suzuki et al., 2000).

In contrast to the broad distribution of *ET_A* expression, *ET_B*

expression in the embryonic heart was predominantly localized to atria and the left ventricle (Fig. 2J-N). Significant hybridization signals of ET_B were already detectable in the E3 heart tube (Fig. 2J). By E6, robust ET_B signals became localized to the atrium and trabeculae of the left ventricle (Fig. 2B), while signals in the right ventricle were indistinct from those of the background. The evident expression of ET_B in the atria was observed throughout heart development (Fig. 2K-N). Although an asymmetric expression of ET_B in the ventricle was detectable until later developmental stages, hybridization signals in the left ventricle declined gradually during heart development (Fig. 2K-N).

Hybridization signals of ET_{B2} transcripts were not evident until E10 (Fig. 2O,P). At this time, signals slightly above background were detected only in the atrioventricular valve leaflets (Fig. 2Q,R). ET_{B2} expression in the valve leaflets increased considerably at later stages of development (Fig. 2S,T). Neither atrial nor ventricular myocardium exhibited overt ET_{B2} signals during heart development. These results clearly show that, of three receptor isotypes, ET_A and ET_B are predominant forms expressed in the myocardium where Purkinje fiber differentiation takes place. The data also demonstrate that ET_A and ET_B expression dramatically changes in the embryonic heart during Purkinje fiber differentiation. The significant decline in hybridization signals of ET_A and ET_B transcripts in the mature

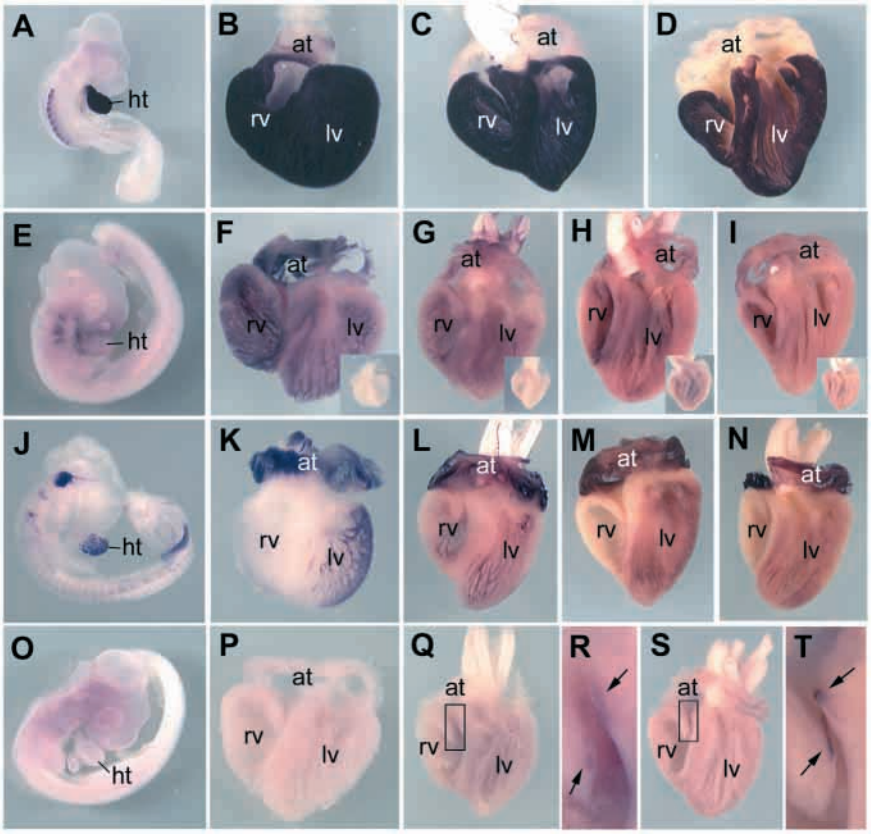

Fig. 2. Whole-mount in situ hybridization of embryonic hearts with antisense riboprobe for VMHC1 (A-D), ET_A (E-I), ET_B (J-N) and ET_{B2} (O-T), and with sense probe (insets in F-I). Whole embryos at E3-E3.5 (A,E,J,O) and whole hearts cut frontally into half at E6 (B,F,K,P), E10 (C,G,L,Q), E15 (H,M,S) and E18 (D,I,N) were examined. Purple staining represents positive hybridization signals. (R,T) High power views of the boxed area in Q and S, respectively. Arrows indicate ET_{B2}-signals in valve leaflets. ht, heart tube; at, atrium; rv, right ventricle; lv, left ventricle.

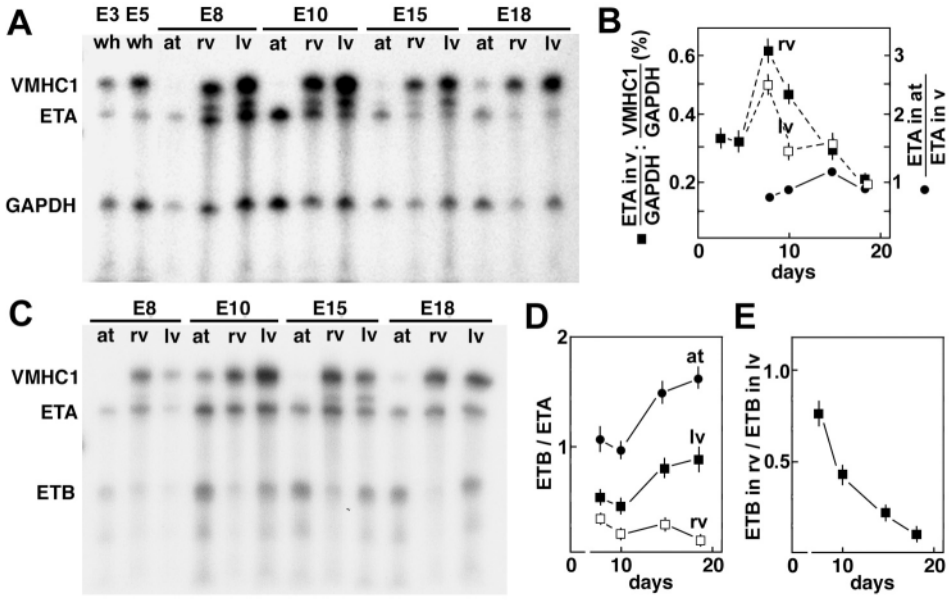

Fig. 3. RNase protection assay of ET_A and ET_B mRNAs expressed in embryonic chick hearts at various stages during development. (A) Autoradiogram of ^{32}P-labelled probes protected by ET_A, VMHC1 and GAPDH mRNAs from whole heart (wh), atrium (at), right ventricle (rv) and left ventricle (lv). VMHC1 and GAPDH served as internal controls to normalize the data of ET receptor mRNAs. Developmental stages are indicated by days of incubation. (B) Quantitation of ET_A mRNA levels expressed in ventricular and atrial chambers during heart development. The data from three different experiments were normalized and averaged. (C) Autoradiograms of RNase protection assay for ET_A and ET_B mRNAs. (D) Quantitation of changes in expression levels of ET_A and ET_B mRNAs in atrial and ventricular chambers during Purkinje fiber differentiation. The data are presented as the ratio of ET_B mRNA to ET_A mRNA. (E) Left ventricle-dominant expression of ET_B in embryonic hearts. Bars indicate standard deviation.

heart suggest that the level of ET-receptor expression is regulated differentially at and after the onset of Purkinje fiber differentiation.

Dynamic changes in ET_A and ET_B transcription in the developing heart

Although the above whole-mount in situ hybridization data show profound changes in distribution of ET_A and ET_B expression in the developing heart, the exact level of their transcription during Purkinje fiber differentiation in the ventricular myocardium was uncertain. Therefore, the amount of ET_A and ET_B mRNAs was examined by RNase protection assay in a chamber-specific manner at various stages of heart development (Fig. 3). Before definitive ventricular partitioning into left and right chambers, the ET_A expression in the myocardium was unclear in our in situ hybridization analysis (Fig. 2), yet RNase protection assay clearly revealed ET_A transcripts as early as the first week of development (Fig. 3A). In the second week of development when hearts had completed ventricular septation, ET_A transcripts were detected in all chambers: atria, and right and left ventricules. This ubiquitous expression pattern of ET_A was maintained throughout heart development (Fig. 3A).

The amount of ET_A mRNAs present in each chamber was then quantified and normalized as a ratio to the amount of *VMHC1* and/or *GAPDH* mRNAs (Fig. 3B). Consistent with the results of in situ hybridization analysis, transcriptional levels of ET_A in the ventricular myocardium increased in the first week of development and peaked at E8, just before the initiation of overt Purkinje fiber differentiation. After this time point, the levels of ET_A mRNAs declined gradually throughout the second and third weeks of development. Similar levels of ET_A mRNAs were detected in the atrium through this developmental window (Fig. 3B). These data clearly show that in the embryonic heart, ET_A is expressed ubiquitously in all chambers and that its expression declines significantly as Purkinje fiber differentiation proceeds.

Transcriptional levels of ET_B were examined in each cardiac chamber (Fig. 3C). In contrast to the ubiquitous expression of ET_A in the heart, ET_B transcripts were predominantly detected in the atrial and left ventricular chambers, while signals only slightly above background levels were detected in the right ventricular chamber. The amount of ET_B mRNAs in each chamber was measured and compared with that of ET_A mRNAs (Fig. 3D). In the atrium, ET_B transcripts were detected at levels comparable with ET_A during the second week of development. This expression became higher than ET_A expression by approximately 50% in the third week. In the left ventricle, ET_B expression was detected about half that of ET_A expression during the second week, and increased to similar levels in the third week. The level of ET_B expression in the right ventricle remained less than 20% of ET_A expression throughout the developmental stages examined. This left-side dominant expression of ET_B in the ventricle became more prominent as embryos developed (Fig. 3E). The results of this RNase protection assay show that in the left ventricle both ET_A and ET_B are expressed at the time of Purkinje fiber differentiation, while ET_A is the dominant form of ET-receptor expressed in the right ventricle. Importantly, these data also show that the total levels of ET receptor expression in the ventricular myocardium decline significantly after the second week of development.

Decreased ET-receptor expression associated with the loss of ET-induced Purkinje fiber differentiation

The above in situ hybridization and RNase protection assay data show that both ET_A and ET_B are expressed in the ventricular myocardium of the embryonic heart. This broad distribution of ET-receptor expression was consistent with our previous studies showing that sites of Purkinje fiber differentiation within the embryonic ventricle are defined by localizing the expression of ECE1, an enzyme responsible for ET ligand production, rather than by localization of expression of ET receptors (Takebayashi-Suzuki et al., 2000). Confirming our previous studies, *ECE1* expression in the ventricle during the first week of development was insignificant except for robust expression in atrioventricular valve leaflets, but became prominent in the endocardium and developing arteries in the second week (Fig. 4A-D). Staining for sMyHC, a standard molecular marker of chick Purkinje fibers, revealed that just after initiation of this spatially restricted expression of *ECE1* in both endocardial and arterial beds in the ventricle,

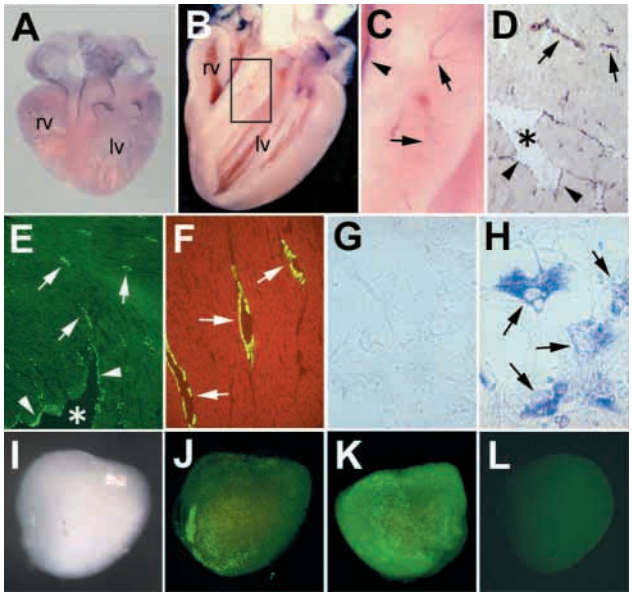

Fig. 4. Expression pattern of ECE1 and conduction cell markers in embryonic chick hearts (A-F), in monolayer (G,H) and organ culture of myocytes (I-L). (A-C) Whole-mount in situ hybridization of E7 (A) and E13 (B) hearts for ECE1. (C) High power view of the boxed area in B. (D) In situ hybridization on a frozen section of E13 ventricle with antisense ECE1 probe. Purple staining in C,D is positive hybridization signal. Arrows and arrowheads in C,D indicate ECE1-positive arterial and endocardial endothelial cells, respectively. Asterisks in D,E indicate the lumen of ventricular chamber. (E) Immunostaining of a frozen section of E15 ventricle with ALD58 (green signal), a Purkinje fiber marker. (F) Same as E but double immunostaining with ALD58 (green signal) and MF20 (red signal), which detects all sarcomeric myosins. Arrows and arrowheads indicate ALD58-positive periarterial and subendocardial Purkinje fibers, respectively. (G) Monolayer culture of myocytes isolated from E3 heart tube. (H) Same as G but exposed to 10^{-7} M ET1 for 5 days just after isolation. Blue staining is ALD58-positive signals (arrows). (I) A ventricular segment isolated from E3 heart tube. (J-L) E3 ventricular segment cultured and (J) stained with ALD58, (K) exposed to 10^{-7} M ET-1 just after isolation and (L) exposed to ET 1 day after isolation. Green signals in J-L are ALD58-positive staining.

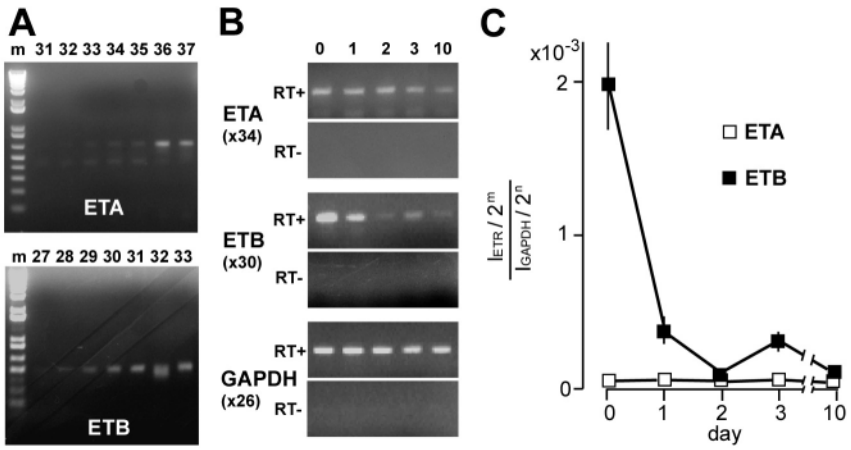

Fig. 5. RT-PCR analysis of ET_A and ET_B mRNAs in heart tubes during organ culture. (A) Amplification of ET_A (top) and ET_B (bottom) cDNA from heart tubes just after isolation with various cycles of PCR. The number of cycles are indicated. m, molecular weight marker. (B) RT-PCR analysis of ET_A and ET_B expression during organ culture. The number of days in culture are indicated. GAPDH served as internal control to normalize the data. RT+, RT-PCR with reverse transcriptase. RT-, RT-PCR without reverse transcriptase. (C) Changes in ET_A and ET_B expression during organ culture. RT-PCR signals of ET receptors (I_{ETR}) and GAPDH (I_{GAPDH}) were normalized by an expected degree of PCR amplification, 2^m and 2^n, respectively. The data are presented as a ratio of ET receptor signal to GAPDH signal from three independent analyses. Bars indicate standard deviation.

juxtaposed subendocardial and periarterial cardiomyocytes differentiated into conduction cells (Fig. 4E,F).

Production of biologically active ET ligands to induce Purkinje fiber differentiation in vivo requires *ECE1* expression (Takebayashi-Suzuki et al., 2000). This processing step of ET ligand production by ECE1 can be bypassed in vitro by exposing embryonic ventricular myocytes directly to synthesized ET ligands (Gourdie et al., 1998). Consistent with these previous studies, addition of synthesized ET ligands induced ventricular myocytes to differentiate into Purkinje fibers both in monolayer (Fig. 4G,H) and whole organ (Fig. 4I-K) cultures. Importantly, however, this inductive response of embryonic myocytes was lost when ventricular myocytes were maintained in culture prior to addition of ET ligand (Fig. 4L). Therefore, it was suspected that expression of ET receptors may decline during culture, as seen in the embryonic heart during development. To test this possibility, the levels of ET_A and ET_B transcripts were examined with RT-PCR analysis during organ culture of E3 heart tubes (Fig. 5). Under our RT-PCR conditions, ET_A transcripts in freshly isolated heart tubes were detectable after 33 cycles of PCR and the signals became saturated after 36 cycles (Fig. 5A). PCR signals of ET_B transcripts were already detectable by 28 cycles and reached a plateau at 31-33 cycles (Fig. 5A). Consistent with the result of in situ hybridization and RNase protection assay (Figs 2, 3), the data show that ET_B is a predominant form of ET receptors expressed in the E3 heart tube, exceeding the level of ET_A expression by over 20- to 30-fold. Throughout the culture period examined, ET_A expression remained at a low level (Fig. 5B,C). In striking contrast, ET_B expression declined dramatically to approximately 10% of the original level within one day of culture and the decreased level of ET_B expression persisted throughout the course of culture (Fig. 5B,C). These results clearly show that the total level of ET-receptor expression in the heart tube drops sharply in culture. The data suggest that this significant decline of ET-receptor expression may be a molecular component responsible for the quick loss of inductive responsiveness of myocytes in culture.

Induced upregulation of ET-receptor expression enhances Purkinje fiber differentiation

We tested whether the expression of exogenous ET receptor by ventricular myocytes, in which endogenous ET-receptor expression has been downregulated, is capable of restoring the ET-dependent differentiation into Purkinje fibers. A replication-defective retroviral vector was engineered to constitutively co-express ET_A and a reporter β-galactosidase (β-gal) (Fig. 6A). The vector was constructed by inserting a ET_A cDNA upstream of an internal ribosome entry sequence of a viral vector, CXIZ. The resulting viral vector was designated CXIZ-ETA. A viral vector encoding only β-gal

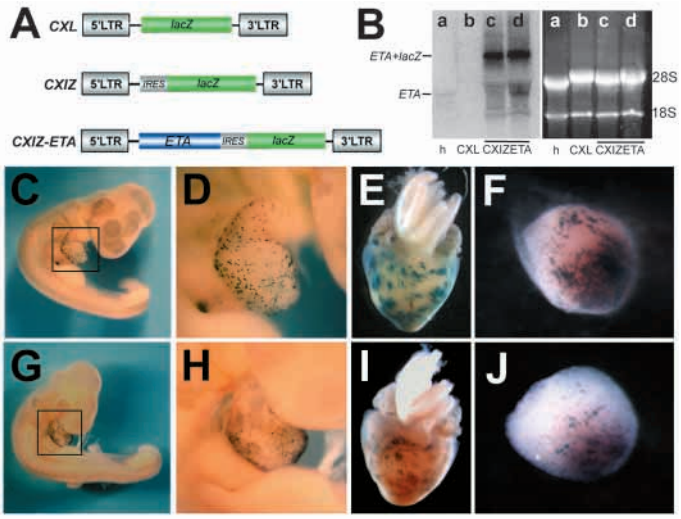

Fig. 6. ET_A-expression retrovirus and in ovo infection. (A) Proviral structures of control viruses, CXL and CXIZ (Mikawa et al., 1992; Mima et al., 1995) and ET_A expression virus, CXIZ-ETA. LTR, long terminal repeat; IRES, internal ribosome entry site; *lacZ*, β-galactosidase gene. (B) Northern blot of ET_A expression from CXIZ-ETA. Total RNAs (20 μg) from E10 heart (lane a), cells transduced with CXIZ (lane b), and cells transduced with CXIZ-ETA (lanes c and d) were separated on agarose gel, stained with Ethidium Bromide (right panel), hybridized with probe for ET_A and autoradiographed (left panel). Endogenous ET_A and heterogeneous ET_A fused to IRES and *lacZ* are indicated. 28S, 28S ribosomal RNA; 18S, 18S ribosomal RNA. (C,G) E4 embryos infected with (C) CXIZ and (G) CXIZ-ETA at E3. (D,H) High power views of the boxed areas in C,G. (E,I) E15 hearts infected with (E) CXIZ and (I) CXIZ-ETA. (F,J) Ventricular segments infected with (F) CXIZ and (J) CXIZ-ETA at E3, isolated at E4, and cultured for 5 days. Blue staining in C-J indicates β-gal positive cells infected with virus.

(CXL) was used as control. Expression of the di-cistronic construct was assured by Northern blot analysis (Fig. 6B). A faint band of approximately 3.5 kb, the expected size of endogenous ET_A mRNA, was detected in the heart. In cells transduced with CXIZ-ETA vector, a band of 6 kb, which was the right size of di-cistronic mRNA encoding *ET_A* and *lacZ*, was detected, whereas no significant hybridization signal was found in cells transduced with control CXL vector (Fig. 6B). The results clearly show the high level of transcription of the ET_A-containing di-cistronic gene in cells transduced with CXIZ-ETA vector.

The recombinant viral particles were introduced into the ventricular myocardium at E3 in ovo and the resulting hearts were examined by staining with X-gal for reporter β-gal at the various developmental stages. As seen in control hearts infected with CXL-virus (Fig. 6C-F), hearts infected with CXIZ-ETA virus exhibited clusters of β-gal-positive cells in the myocardium within 24 hours after injection (Fig. 6G,H) and maintained the expression of exogenous gene until later in development (Fig. 6I). This stable expression of *ET_A*-containing transgene was also found in virus-infected ventricular segments in culture (Fig. 6F,J) as seen in those infected with control CXL virus. The data demonstrate the high infectivity of embryonic myocytes with CXIZ-ETA-virus comparable with CXL virus, and the stable expression of introduced exogenous genes during in ovo cardiogenesis and in organ culture.

To examine the effect of constitutively expressed ET_A, virus-infected ventricular segments in which the expression of endogenous ET-receptors had been already downregulated (Fig. 5) were exposed to ET-1 peptide in culture (Fig. 7). Infected cells and Purkinje fiber marker-positive cells were identified by double-immunostaining for β-gal and sMyHC, respectively, in wholemount followed by histological sectioning. Under our culture condition, CXL virus-infected ventricular segments were rarely induced by the addition of ET-1 to express the conduction cell marker ALD58 (Fig. 7A-F). Inspections in whole mount and on histological sections failed to detect significant induction of Purkinje fibers from uninfected ventricular cells (Fig. 4L). By contrast, CXIZ-ETA virus-infected ventricular segments displayed robust signals of the conduction cell marker in response to added ET-1 (Fig. 7G-L). Frequency of this ET-dependent induction of Purkinje fiber-specific signal in infected cells was significantly higher in CXIZ-ETA virus-infected hearts than in those infected with control CXL virus (Fig. 7M). These results suggest that the constitutive expression of exogenous ET_A is capable of maintaining and/or enhancing the inductive responsiveness of ventricular myocytes to ET1 ligand to differentiate into Purkinje fibers.

DISCUSSION

Our previous studies demonstrated that Purkinje fibers differentiate from myocytes (Gourdie et al., 1995; Cheng et al., 1999) and that this terminal differentiation event in the myocyte lineage can be induced by paracrine signal(s) from cardiac vascular cells (Hyer et al., 1999), such as ET (Gourdie et al., 1998; Takebayashi-Suzuki et al., 2000). The activation site of the inductive signaling in the embryonic heart is spatially confined by the localized expression of *ECE1* in the endocardial and arterial endothelium (Takebayashi-Suzuki et al., 2000). Importantly, the ability of ET-dependent transdifferentiation into conduction cells is gradually lost from myocytes as heart development proceeds (Gourdie et al., 1998), and mature myocytes undergo hypertrophy rather than conduction cell differentiation in response to the ET signal (Ito et al., 1991). The present study addressed the potential mechanism(s) underlying the developmentally regulated loss of ET-induced Purkinje fiber differentiation. The data presented show dynamic changes in expression of three ET receptor isotypes in the heart during chick embryogenesis. Importantly, downregulation of endogenous ET-receptor expression appeared to be associated with the age-dependent loss of ET-induced conduction cell differentiation from myocytes. The expression of exogenous ET receptor in myocytes, in which endogenous receptors had been downregulated, resulted in restoration and/or maintenance of their ET-dependent ability to differentiate into conduction cells. These results suggest that the level of ET-receptor expression is a factor responsible for the developmentally regulated responsiveness of embryonic myocytes to ET signaling to differentiate into Purkinje fibers.

Three isotypes of ET receptor, ET_A, ET_B and ET_{B2}, have been identified in the avian (Kempf et al., 1998; Lecoin et al., 1998; Nataf et al., 1996). As no specific antibodies for individual ET receptor isotypes of the chick are currently

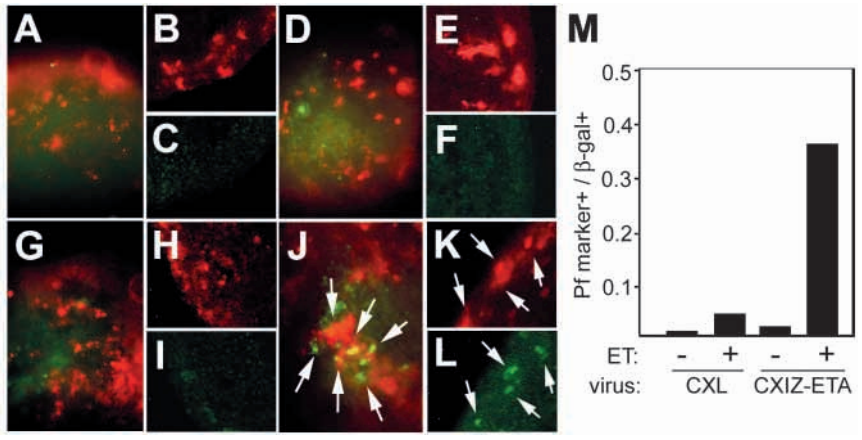

Fig. 7. Ectopic induction of Purkinje fiber marker by the expression of exogenous ET_A. CXIZ- (A-F) or CXIZ-ETA- (G-L) infected heart tubes were removed from embryos, and ventricular segments were isolated and cultured in the absence (A-C,G-I) or the presence (D-F,J-L) of 10^{-7} M of active ET-1 peptide. Tissues were double immunolabeled in whole mount with anti-β-gal antibody (red signal) and ALD58 (green signal). Immunolabeled ventricular segments (A,D,G,J) were further processed for frozen sectioning (B,C,E,F,H,I,K,L). (B,E,H,K) β-gal and (C,F,I,L) ALD58 signals in the same cultures. Arrows indicate cells positive for conduction cell marker ALD58. (M) Ratio of ALD58-positive cells per viral infected cells. In each experimental group, a total of 50-100 cells from five infected hearts were examined and averaged.

available, little is known about the expression patterns of these receptor isotypes in the embryonic heart during conduction system development (Watanabe et al., 1989; Clouthier et al., 1998; Yanagisawa et al., 1998; Takebayashi-Suzuki et al., 2000). It has been uncertain which receptor isotype(s) is responsible for ET-induced Purkinje fiber differentiation. To address this question, three chick cDNAs specific for each receptor isotype were cloned in the present study. Deduced amino acid sequences from these cDNA clones show a 100% match of our ET_A clone with the published chick ET_A sequence (Nataf et al., 1996) and over 95% homology to the cloned chick ET_B and ET_{B2} with quail homologs (Lecoin et al., 1998). While both chick ET_A and ET_{B2} cDNAs encode a full coding sequence, ET_B cDNA is a partial cDNA lacking the translation initiation site. Thus, the exact identity of the chick ET_B clone awaits the cloning of the full-length cDNA. However, the high homology of its sequence with quail ET_B, including all the structural characteristics of the G protein-coupled ET-receptor family (Arai et al., 1990; Sakurai et al., 1990), suggests that the chick clone probably represents the ET_B sequence.

Using these probes, the present study showed that ET_A is expressed broadly throughout the embryonic chick heart, prior to and during Purkinje fiber differentiation. While ET_B expression is also found in both the ventricular and atrial myocardium in the same developmental window, it is expressed at a significantly higher level in the atrium and left ventricle than in the right ventricle. By contrast, ET_{B2} expression appears to be primarily confined to developing AV-valve leaflets. Although the molecular basis for the isotype-specific expression pattern of ET receptors in the embryonic heart is currently unknown, the data suggest that the expression of each receptor isotype is regulated independently and may play distinct roles in the embryonic heart. This idea is consistent with differential expression and function of ET_A and ET_B along the anteroposterior axis in the mouse (Arai et al., 1990; Yanagisawa et al., 1998). Nevertheless, there seems an obvious difference in receptor expression pattern between chick and mouse embryos. Prior to overt Purkinje fiber differentiation, ET_B expression in the chick embryonic heart appears to predominate over ET_A expression in the ventricular myocardium. While this result is consistent with our previous studies (Gourdie et al., 1998) that ET-induced conversion of E3 chick myocytes is inhibited more effectively by an ET_B-specific antagonist than an ET_A-antagonist, no significant heart defects have been identified in the mouse and human in which the genes for ET_B are deleted or mutated (Hosoda et al., 1994; Baynash et al., 1994). Future studies should reveal the evolutionary significance of different expression patterns among receptor isotypes in different species.

Our data show that ET_A and ET_B are dominant isotypes expressed in the ventricular myocardium during Purkinje fiber differentiation, while ET_{B2} does not seem to be a major receptor type responsible for ET-dependent Purkinje fiber differentiation. The significance of ET_{B2} expression in developing valve leaflets remains to be addressed. Interestingly, ET_A and ET_B expression in the ventricle exhibits a biphasic pattern during heart development with the peak during the second week of incubation. The peak of expression of these two receptor isotypes coincides with initiation of overt Purkinje fiber differentiation (Gourdie et al., 1995; Takebayashi-Suzuki et al., 2000). For example, the upregulation of an early conduction cell marker Cx42 becomes detectable at this time point in presumptive Purkinje fibers, which are differentiated from ventricular myocytes along developing arterial beds (Rychter and Ostadal, 1971; Mikawa and Gourdie, 1996) and the endocardium as seen in the present study. While the expression of *ECE1* remains highly active in atrial and endocardial endothelial cells throughout heart development (Takebayashi-Suzuki et al., 2000), the present study shows that the expression of ET_A and ET_B declines significantly after this developmental stage. A sharp decline of receptor expression is also the case in heart tubes soon after they are transferred to organ culture, leading to the loss of myocyte responsiveness to mature ET to differentiate into Purkinje fibers. In contrast to *ECE1* expression, which spatially confines the site of Purkinje fiber differentiation in the ventricle (Takebayashi-Suzuki et al., 2000), the close association between receptor expression and inductive responsiveness of myocytes suggests that the level of ET receptors may define the ability of myocytes to undergo ET-induced conversion to Purkinje fibers.

This idea is further supported by a successful restoration of ET-induced expression of sMyHC, a marker for fully differentiated conduction cells, by introducing an exogenous ET_A expression into myocytes via virus infection. The recombinant virus used in the present study is designed to co-express ET_A and a reporter β-gal to detect infected cells, as no specific antibody to the chick ET_A is currently available and it is undetermined whether physiological functions of ET receptor remain unchanged by tagging with an epitope. Thus, the exact level of ET_A protein in individual infected cells has not been determined in the present study. However, the data of our northern blot analysis show that CXIZ-ETA virus-infected cells produce a large amount of dicistronic mRNAs encoding ET_A and β-gal. We have previously shown that two protein

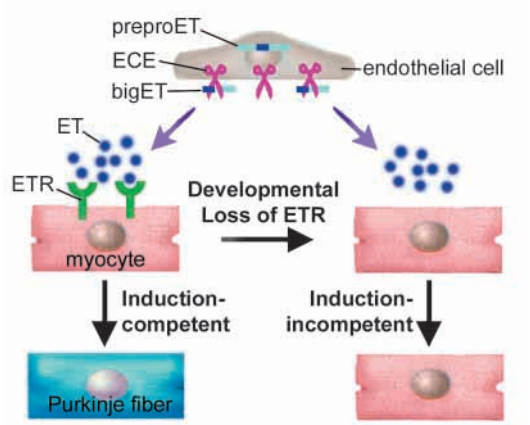

Fig. 8. Model for age-dependent differentiation of Purkinje fibers from embryonic myocytes induced by local production of ET ligand and its interaction with ET receptor. In the embryonic chicken heart, there are two types of endothelial cells: ECE-expressing and non-expressing cells. Only the former convert bigET into mature ET. Among ET receptor (ETR)-positive myocytes, only those adjacent to ET production can be induced to differentiate into conduction cells. As embryonic development proceeds, the level of ET receptors in myocytes declines, resulting in the loss of competency to respond to ET ligand and to differentiate into conduction cells.

species are equally co-translated from a dicistronic mRNA in infected cells (Mima et al., 1995; Itoh et al., 1996; Lin et al., 1999; Das et al., 2000). Furthermore, sMyHC induction is preferentially found in cells infected with CXIZ-ETA virus, but not in those infected with control CXL virus. Therefore, we conclude that ET-dependent Purkinje fiber differentiation seen in CXIZ-ETA virus-infected cells is likely dependent upon upregulated ET_A expression. It should be noted, however, that ET-dependent sMyHC expression is induced only in a subpopulation of infected cells. It remains to be determined whether this differential response among CXIZ-ETA virus-infected cells results from variation in ET_A synthesis, or that of other downstream components involved in the ET-signaling cascade.

As a Purkinje fiber network is vital for pacemaking the rhythmic heart beat, dysfunction of this essential tissue often leads to arrhythmias and conduction block. Regeneration and/or repair of the cardiac conduction network has not been considered feasible. Our previous studies have demonstrated that embryonic myocytes have the potential to undergo differentiation into Purkinje fibers in response to a paracrine signal both in vivo and in vitro. The present study now indicates that this paracrine signal-dependent conversion of contractile myocytes into conduction cells can be restored and/or enhanced at least partly by upregulating a membrane receptor specific to the inductive paracrine signal. The expression and function of ET receptors presented in this study, together with our previous studies of ECE1 (Takebayashi-Suzuki et al., 2000), are consistent with the model (Fig. 8) in which ET-induced Purkinje fiber differentiation is spatially and temporally regulated in the ventricular myocardium during chick heart development. In addition to furthering our understanding of the development of specialized myocardial tissues, this study may provide a new approach for replacing and/or repairing dysfunctional tissue of the cardiac conduction system.

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