

# Hemodynamic-dependent patterning of endothelin converting enzyme 1 expression and differentiation of impulse-conducting Purkinje fibers in the embryonic heart

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Accepted 27 October 2003

Development 131, 581-592  
Published by The Company of Biologists 2004  
doi:10.1242/dev.00947

## Summary

Impulse-conducting Purkinje fibers differentiate from myocytes during embryogenesis. The conversion of contractile myocytes into conduction cells is induced by the stretch/pressure-induced factor, endothelin (ET). Active ET is produced via proteolytic processing from its precursor by ET-converting enzyme 1 (ECE1) and triggers signaling by binding to its receptors. In the embryonic chick heart, ET receptors are expressed by all myocytes, but ECE1 is predominantly expressed in endothelial cells of coronary arteries and endocardium along which Purkinje fiber recruitment from myocytes takes place. Furthermore, co-expression of exogenous ECE1 and ET-precursor in the embryonic heart is sufficient to ectopically convert cardiomyocytes into Purkinje fibers. Thus, localized expression of ECE1 defines the site of Purkinje fiber recruitment in embryonic myocardium. However, it is not known how ECE1 expression is regulated in the embryonic heart. The unique expression pattern of ECE1 in the embryonic heart suggests that blood flow-induced stress/stretch may play a role in patterning ECE1

expression and subsequent induction of Purkinje fiber differentiation. We show that gadolinium, an antagonist for stretch-activated cation channels, downregulates the expression of ECE1 and a conduction cell marker, Cx40, in ventricular chambers, concurrently with delayed maturation of a ventricular conduction pathway. Conversely, pressure-overload in the ventricle by conotruncal banding results in a significant expansion of endocardial ECE1 expression and Cx40-positive putative Purkinje fibers. Coincident with this, an excitation pattern typical of the mature heart is precociously established. These *in vivo* data suggest that biomechanical forces acting on, and created by, the cardiovascular system during embryogenesis play a crucial role in Purkinje fiber induction and patterning.

Key words: Cardiac conduction system, Optical mapping, Hemodynamics, Mechanosensor, Conotruncal banding, Gadolinium, Connexin 40, Chick embryo

## Introduction

A rhythmic heartbeat is coordinated by the pacemaking action potentials of the cardiac conduction system (Tawara, 1906; Bozler, 1942). The pacemaking impulses are evoked at the sinoatrial node and conducted across the atrial chambers, causing atrial contraction. At the atrioventricular junction, the pacemaking impulses do not directly spread from atrial muscle into ventricular muscle. Instead, impulse is slowed within the atrioventricular node and then rapidly propagated down the interventricular septum, along the atrioventricular bundle and its branches. From the bundle branches, the pacemaking impulses spread along the most distal, fast conduction component, the Purkinje fiber network, and are finally transmitted to ventricular muscle via a so-called Purkinje-myocyte junction concentrated at the apex of the heart. Thus,

this specialized pathway of impulse conduction is the mechanism for the apex-to-base wave of excitation and contraction of the ventricular myocardium of the mature heart (reviewed by Mikawa and Fischman, 1996; Moorman et al., 1998; Gourdie et al., 1999).

The impulse conduction pathway of the heart is established during embryogenesis through a complex and dynamic process. Electrode and optical mapping studies of embryonic chick hearts have demonstrated that when the primitive heart tube forms, all epithelioid myocytes are electrically active, but pacemaking impulses are evoked predominantly by myocytes in the posterior inflow tract (Kamino et al., 1981; Kamino, 1991; de Jong et al., 1992). These impulses spread to the anterior end of the heart, towards the outflow tract, via gap junctions between the epithelioid myocytes without any local

changes in velocity. Therefore, except for the pacemaker cells, no other subcomponents of the conduction system are detected at this stage. Soon after the heart tube loops, a change of the impulse propagation pattern along the myocardium becomes detectable. Impulse velocity becomes significantly slowed at the atrioventricular junction and is much faster through the ventricle (Lieberman and Paes de Carvalho, 1967; de Jong et al., 1992), suggesting that both slow and fast conduction components have begun to differentiate (Moorman et al., 1998). This unidirectional activation sequence across the ventricle from the AV junction towards the outflow tract remains during heart looping. As the atrioventricular bundle, bundle branches and Purkinje fibers fully develop and are linked as an entire ventricular conduction network, the activation sequence of the ventricle undergoes a dramatic topological shift from an immature, unidirectional pattern to the mature, apex-to-base pattern (Chuck et al., 1997; Reckova et al., 2003).

Although little is known about how these distinct conducting elements are induced, patterned and integrated into an entire conduction system network, significant progress has been made in the last few years in our understanding of cellular and molecular mechanisms that regulate the differentiation of Purkinje fibers (reviewed by Mikawa and Fischman, 1996; Mikawa, 1999a; Mikawa, 1999b; Gourdie et al., 1999; Pennisi et al., 2002). In the chicken, the Purkinje fiber network develops subendocardially and penetrates intramyocardially along branching coronary arteries (Pattern and Kramer, 1933; Vassal-Adams, 1982; Gourdie et al., 1995; Cheng et al., 1999; Takebayashi-Suzuki et al., 2000). Purkinje fibers can be identified by their unique gene and protein expression patterns (reviewed by Schiaffino, 1997; Moorman et al., 1998; Welikson and Mikawa, 2001). Our retroviral cell lineage studies have further shown that cells of individual conducting elements locally differentiate from working myocytes, not by outgrowth from a prespecified common progenitor (Gourdie et al., 1995; Cheng et al., 1999). Importantly Purkinje fiber recruitment takes place exclusively along the developing endocardium and coronary arterial branches, but not veins or capillaries. Thus, Purkinje fiber differentiation in the embryonic heart is tightly regulated both temporally and spatially (Mikawa, 1999a; Mikawa, 1999b; Gourdie et al., 1999; Pennisi et al., 2002). This phenotype conversion is induced by vessel-derived paracrine signals (Hyer et al., 1999), including the stretch/pressure-induced factor, endothelin (ET) (Gourdie et al., 1998; Takebayashi-Suzuki et al., 2000; Takebayashi-Suzuki et al., 2001). These findings raised the question of how the sites and timing of Purkinje fiber differentiation are precisely defined in developing hearts using ET as an inductive signal.

Active ET (Yanagisawa et al., 1988) is secreted after proteolytic processing from its precursor by ET-converting enzyme 1 (ECE1) (Xu et al., 1994) and signals by binding to its receptors (Arai et al., 1990; Sakurai et al., 1990). In the embryonic chick heart, two ET receptors, ET<sub>A</sub> and ET<sub>B</sub> (Nataf et al., 1996; Nataf et al., 1998; Lecoin et al., 1998), are ubiquitously expressed by cardiomyocytes (Kanzawa et al., 2002). By contrast, ECE1 is expressed in a subset of endocardial endothelia and all arterial endothelial cells, but not by veins or capillaries (Takebayashi-Suzuki et al., 2000). Thus, the expression of endogenous ECE1 is restricted to the sites where adjacent myocytes are induced to differentiate into

Purkinje fibers. Furthermore, co-expression of exogenous ECE1 with ET precursor (preproET), but not ET precursor expression alone, in the embryonic heart is sufficient to ectopically convert myocytes to Purkinje fibers (Takebayashi-Suzuki et al., 2000). Therefore, spatial restriction of ECE1 expression is key for patterning the differentiation of the cardiac Purkinje fiber network. It is unknown, however, how the expression of endogenous ECE1 is regulated in the embryonic heart.

Many studies have shown that hemodynamic forces, such as shear stress and/or stretch, can regulate the expression of preproET and production of mature ET in cultured endothelial cells (Yoshizumi et al., 1989; Malek and Izumo, 1992; Wang et al., 1993; Macarthur et al., 1994; Zhu et al., 1997; Morawietz et al., 2000; Garcia-Cardena et al., 2001). It has recently been shown that blood pressure changes in chick embryonic hearts can influence conduction system development (Reckova et al., 2003). Furthermore, the expression of ECE1 in the embryonic ventricle is restricted to endocardial and arterial endothelial cells (Takebayashi-Suzuki et al., 2000), areas that are exposed to higher shear-stress/stretch than veins and capillaries. Taken together, we hypothesized that ECE1 expression and its ultimate induction of Purkinje fibers are regulated by biomechanical forces such as shear-stress/stretch. Biomechanical force-dependent response is mediated by several mechanosensors (reviewed by Traub and Berk, 1998), including intracellular Ca<sup>2+</sup> ions (Dull and Davies, 1991), a barium-inhibited inward-rectifying shear-sensitive K<sup>+</sup> channel I<sub>KS</sub> (Olesen et al., 1988; Jacobs et al., 1995), a gadolinium-inhibited stretch-activated cation channel I<sub>SA</sub> (Lansman et al., 1987; Yang and Sachs, 1989; Naruse et al., 1998; Suchyna et al., 2000), tyrosine phosphorylation (Takahashi and Berk, 1996) and integrin-mediated signaling (Chen et al., 2001). However, it is still controversial whether biomechanical forces can regulate ECE1 expression in cultured endothelial cells (Harrison et al., 1998; Masatsugu et al., 1998; Morawietz et al., 2000). Furthermore, the role of hemodynamics and biomechanical forces in regulating the timing and location of ECE1 expression in cardiac endothelial cells has not been studied in the embryonic heart *in vivo*.

In the present study, we show that ECE1 expression in the endocardium and Cx40 (also known as Cx42 or alpha 5) expression in the presumptive Purkinje fibers of the embryonic chick heart are both diminished by gadolinium, an antagonist of I<sub>SA</sub>-channels, and are enhanced by increasing pressure load with conotruncal banding. Associated with this, a shift of activation sequences from the immature, unidirectional pattern to the mature, apex-to-base pattern is delayed in gadolinium-treated hearts and precociously induced in pressure-overloaded hearts. These results provide for the first time *in vivo* evidence for the inductive role of hemodynamic(s) on the expression of ECE1 in the embryonic chick heart. Associated changes in Cx40 expression and activation sequence development further suggest that biophysical forces that are generated by the cardiovascular system itself play a crucial role in Purkinje fiber differentiation and patterning.

## Materials and methods

### Embryos

Chick embryos were used in all experiments of the present study.

Fertile chick eggs were purchased from either SPAFAS (Charles River, NJ), Truslow Farms (Chestertown, MD) or ISE (Newberry, SC) and incubated at 38°C under humidified conditions. Embryos were staged according to the number of days of incubation or by the staging system of Hamburger and Hamilton (Hamburger and Hamilton, 1951).

### In situ hybridization

Expression patterns of ECE1 and Cx40 in the embryonic hearts were examined by in situ hybridization analysis as described previously (Takebayashi-Suzuki et al., 2000; Takebayashi-Suzuki et al., 2001; Kanzawa et al., 2002) with slight modifications as follows. In short, formaldehyde-methanol fixed embryonic hearts were treated with 10 µg/ml proteinase K for 10-30 minutes at room temperature, depending on stage or age. After terminating proteinase K reaction with glycine (0.1 g/50 ml PBT) and post-fixation with 4% formaldehyde and 0.1% glutaraldehyde, samples were rinsed twice with PBS, pre-incubated with the hybridization mixture for 1 hour at 65°C, and reacted overnight at 65-70°C with DIG-labeled RNA probes (3.5 µg/ml of ECE1 and 1 µg/ml of Cx40). After 65°C washes and blocking steps, the samples were incubated overnight at 4°C with alkaline phosphatase-conjugated anti-DIG antibody (1:2000 dilution, Roche). After washing, samples were stained with the NBT/BCIP mixture at room temperature for color development. Hearts stained in whole-mount were further processed for paraffin sectioning as described previously (Takebayashi-Suzuki et al., 2000).

### Optical mapping

Activation sequences of the embryonic heart ventricle were analyzed by optical mapping techniques as described previously (Kamino, 1991; Litchenberg et al., 2000; Rentschler et al., 2001; Reckova et al., 2003). The basic setup of the detection system is illustrated in Fig. 1A. In brief, hearts were stained with a voltage-sensitive dye [di-4-ANEPPS (Molecular Probes), 0.1 µg/ml] for 5 minutes at room temperature and superfused at 37°C with Tyrode's solution (pH 7.4) saturated in a small oxygen chamber mounted on a Leica DML-FS fluorescent microscope. The spread of excitation across the myocardium was monitored by changes in fluorescence signals of di-4-ANEPPS, using a fast (up to 1300 frames per second at 12 bit resolution) CCD camera (Olympus Neurocam) with an 80×80 pixel array. Excitation light (520±40 nm) was provided by a 100 W mercury vapor light source and a green filter. Cytochalasin D (75 µM) was used to minimize motion artifact (Biermann et al., 1998; Jalife et al., 1998; Reckova et al., 2003). Data were acquired using Ultra-View software (Perkin-Elmer Lifesciences, version 3.0), and analyzed with the Universal Mapping program written by Dr Martin Biermann in the IDL5.4 (RSI, Boulder CO) programming language. Isochronal maps of the activation sequences were generated by marking activation times at the  $dv/dt_{max}$  of the action potential upstrokes in a 40×40 matrix and plotting those times. Isochronal maps and Quick-Time movies were used to determine the activation sequence of each heart.

### In ovo gadolinium injections

Diethylenetriaminepentaacetic acid, Gadolinium III dihydrogen salt hydrate, (GdDPTA) (Sigma Aldrich) was dissolved in Tyrode's saline buffer to make stock solutions of 1 mM, 10 mM and 100 mM and pH was adjusted to 7.0 with NaOH, filtered through a 0.2 µm pore membrane, and aliquoted into 1 ml volumes. To the aliquots, 5 µl of 0.4% Trypan Blue was added, and stored at 4°C. A small hole was made in the blunt end of the egg, and the inner shell membrane was removed to expose the extra-embryonic, vitelline veins. An injection needle was made from a 1.0 mm glass capillary using a vertical pipette puller. Using a Picospritzer 2 micro-injection setup described elsewhere (Fischman and Mikawa, 1997), a volume of 3.0 µl was pulse-injected into the anterior or posterior vitelline vein through which oxygenated blood flow directly returns to the heart via the sinus venosus. Embryos injected with Tyrode's saline without GdDPTA and

those uninjected were used as controls. The injected eggs were sealed with Parafilm and returned to the incubator for various times after injection.

### Conotruncal banding (CTB)

Eggs were incubated as described above for 3.5 days until around HH stage 21. After making a hole of 5-10 mm diameter in the shell, the inner shell membrane was removed from above the embryo. CTB was performed according to Clark et al. (Clark et al., 1989), using 10-0 nylon suture (Sedmera et al., 1999; Tobita et al., 2002). The suture was tied around the conotruncus, but with no constriction of blood flow at the time of operation. Sham-operated embryos underwent the same procedure but had the suture removed immediately. The eggs were sealed with Parafilm and reincubated for additional 3 days until HH stages 27-29.

### RT-PCR analysis

Total RNA from the ventricles was extracted as described previously (Takebayashi-Suzuki et al., 2001; Kanzawa et al., 2002), using TRIzol reagent (Invitrogen), and treated with RNase-free DNaseI (Roche). RNA (200 ng) was converted into cDNAs using AMV Reverse Transcriptase (Roche), Random Hexamers (Roche) and amplified by the Polymerase Chain Reaction (PCR). Primers and PCR conditions were as follows: GAPDH, 5'CAGCCTTCACTACCCTCTTG3' (forward) and 5'ACGCCATCACTATCTTCCAG3' (reverse); ECE1, 5'ACCGCATCTCACCTTCTTC3' (forward) and 5'AGGATAGAA-GACCGTGGAGA3' (reverse); Cx40, 5'GTCCGCCCCACAGG-TAGAAA3' (forward) and 5'GTCCACGGGCTGAGAAGCTT3' (reverse); and VEGF, 5'CAGGCCATCCTGTGTGCCTCT3' (forward) and 5'TTCCGCTGCTCACCGTCTCGG3' (reverse). GAPDH cDNA was amplified by 29 cycles with a 55°C annealing temperature, while VEGF cDNA was by 37 cycles with a 60°C annealing temperature. ECE1 and Cx40 cDNAs were amplified together in a duplex reaction for 37 cycles with a 50°C annealing temperature. PCR products (12 µl) were electrophoresed on a 1% agarose gel and visualized using ethidium bromide. The band intensities were digitally captured by Fluor-S MultiImager and analyzed using Multi-Analyst software (BioRad). The level of GAPDH cDNA was used to normalize the intensity of signal. Intensity data were then subjected to statistical analyses using Microsoft Excel software (Microsoft).

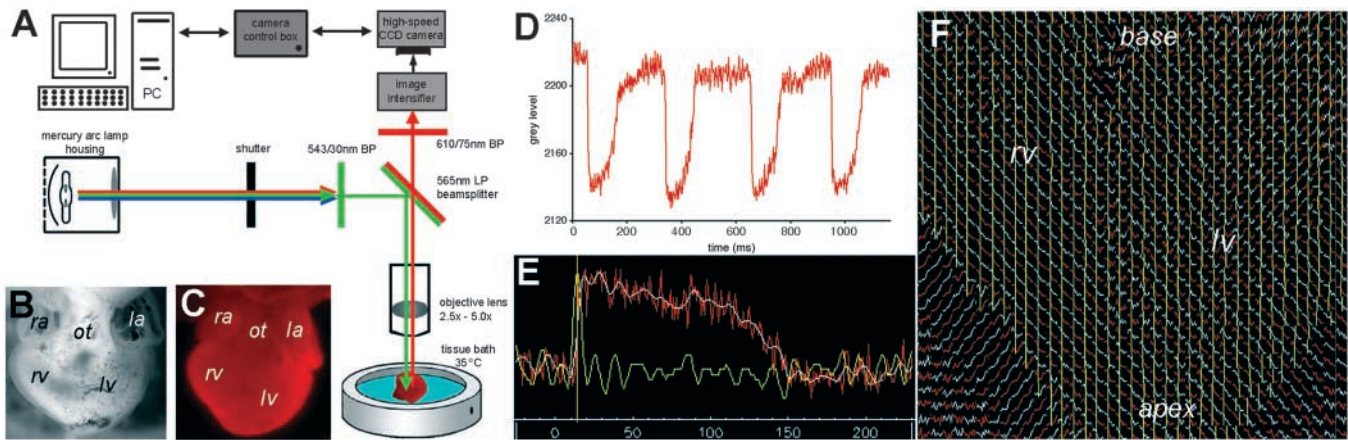
### Data documentation

All images of hearts and sections were captured by either a Digital Photo Camera (DKC-5000, Sony) or a Spot RT Slider (Diagnostic Instruments) using Adobe Photoshop (Adobe Systems) or Spot software. Images were adjusted for color levels, brightness and contrast using Adobe Photoshop software.

## Results

### ECE1 and Cx40 expression patterns during fast conduction pathway maturation

The present study examined the potential role of biomechanical forces on the endocardial expression of ECE1 and its downstream induction of ET-dependent subendocardial Purkinje fiber differentiation during chick embryogenesis. Expression patterns of endocardial ECE1 were mapped by in situ hybridization analysis, while Purkinje fiber differentiation was monitored by both the impulse propagation pattern across the ventricle and by the expression of chick Cx40 (also called Cx42 or alpha 5), a gap-junctional protein thought to be responsible, in part, for the fast conduction properties of Purkinje fiber tissues. In the first set of experiments, we established a spatial map of both endocardial ECE1 and



**Fig. 1.** Optical mapping of electrical activation in the embryonic chick heart. (A) Setup consisting of epifluorescence microscope, temperature-controlled oxygenated organ bath and computer-controlled intensified high-speed camera. (B) Brightfield image of E6.5 heart immediately after recording. (C) Epifluorescence signals of di-4-ANEPPS from the same heart captured by the high-speed camera (80×80 pixels). (D) An example of changes in fluorescence intensity over time from a 4×4 pixel region of the ventricle (raw 12 bit data). Drops in fluorescent intensity level correspond to depolarization. (E) A typical action potential recording from a single pixel that was inverted, digitally filtered (white line), and the first derivative (yellow) calculated. Peak of the first derivative, corresponding to maximum upstroke velocity, was determined (orange) to mark the time of activation of individual pixels. *x* axis scale in milliseconds. (F) An example of two-dimensional array of the optical mapping data. la, left atrium; ra, right atrium; lv, left ventricle; rv, right ventricle.

myocardial Cx40 expression, compared with developmental changes in impulse propagation patterns in the ventricle of control embryonic chick hearts.

Using a standard optical mapping setup (Fig. 1A), the propagation pathway of action potentials across the ventricular wall was analyzed at various stages of development. Embryonic hearts were stained with a voltage sensitive dye di-4-ANEPPS (Fig. 1B,C) as a read-out for changes in the membrane potential. The intensity of fluorescent signals across the ventricular surface was recorded. Action potentials were detected as a rapid decrease of di-4-ANEPPS fluorescent signal (Fig. 1D). The optical records from individual pixels were then inverted and the first derivative of changes in the fluorescence intensity was calculated. The time of activation at individual pixels was determined as the peak of the first derivative, or the maximum upstroke velocity (Fig. 1E). The activation data from all pixels covering the entire ventricular surface (Fig. 1F) were used for generating isochronal maps (Fig. 2A-C) to evaluate the impulse propagation pattern.

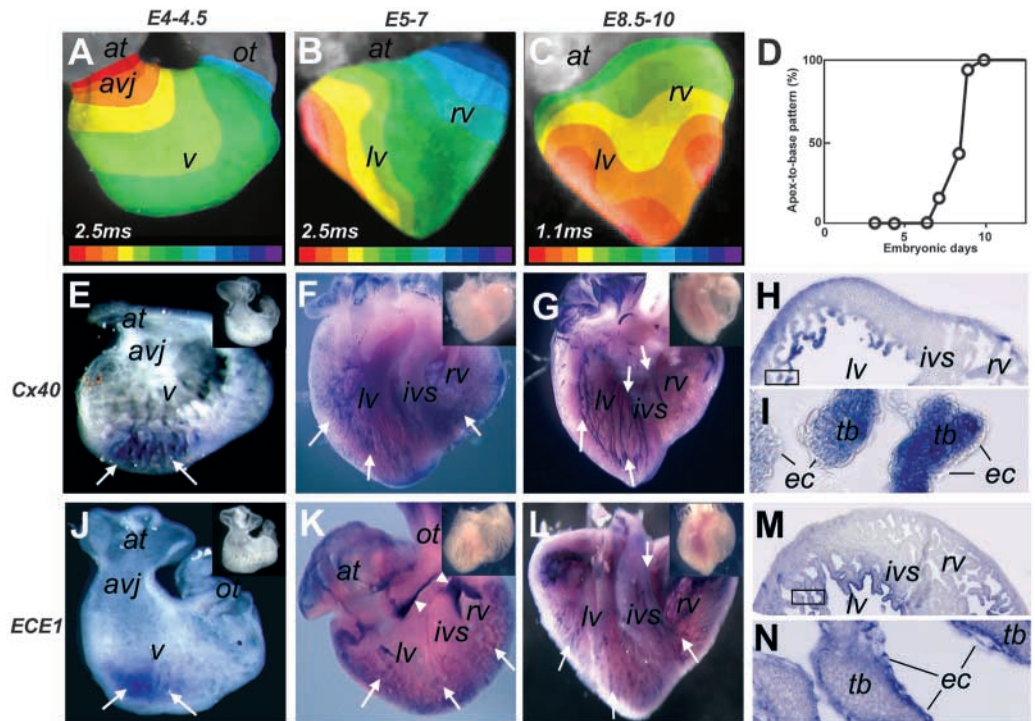
All hearts examined at looping stages at around embryonic days 3-4.5 (E3-4.5) exhibited slower conduction velocity at the atrioventricular junction and faster velocity across the ventricle toward the outflow tract (Fig. 2A,D). (The first activation site is indicated in red.) During early interventricular septum formation at E5-E7, the unidirectional propagation of impulses from the left ventricle through the right ventricle and toward the outflow tract was seen in all hearts examined (Fig. 2B,D). The topological shift from this immature, lateral propagation pattern to the mature, apex-to-base pattern (Fig. 2C) became first detectable in a small population of hearts by E7.5 (Fig. 2D). By E8.5, the stage of complete interventricular septation (Chuck et al., 1997), the majority of hearts showed the apex-to-base activation sequence. By E10, the mature propagation sequence was detected in all hearts examined (Fig. 2D) and in no case was the immature, lateral propagation pattern seen after this developmental stage. The observed timecourse of

activation development between E3 and E10 was consistent with previous electrode and optical mapping studies (de Jong et al., 1992; Chuck et al., 1997; Reckova et al., 2003), and served as a functional read-out of the effect of mechanosensors and hemodynamic forces on Purkinje fiber differentiation and patterning.

Although optical mapping analysis of activation sequences across the ventricular surface was powerful enough to determine the developmental/functional status of the entire conduction system, it did not provide the resolution to specifically identify the developing Purkinje fiber network. Therefore, the localization of differentiating Purkinje fibers in embryonic ventricles during conduction pathway development was determined by examining the expression of a specific Purkinje fiber maker, Cx40, a gap junctional protein thought to be essential for fast conduction of action potentials (Gourdie et al., 1993; Takebayashi-Suzuki et al., 2000; Tamaddon et al., 2000). Coinciding with the initiation of fast conduction in the developing ventricle (Fig. 2A), Cx40 signals were detectable in the ventricle at heart looping stages (Fig. 2E). Importantly, a higher level of hybridization signal was seen in the outer curvature of the ventricle, where action potentials propagated faster than in other regions at this developmental stage (Fig. 2A,E).

By E5-7, Cx40 expression expanded in the ventricle, exhibiting a fine network of hybridization signals along the developing trabeculae (Fig. 2F). However, no specific path of Cx40 staining signals linking the base and apex of the ventricle was observed (Fig. 2F), consistent with the immature lateral activation pattern across the ventricle at this developmental stage (Fig. 2B). During and after the topological shift of activation sequence from the immature pattern to the mature apex-to-base (Fig. 2C), Cx40 expression became more robust and exhibited arrays along the longitudinal axis of the ventricle (Fig. 2G). This staining pattern is typical of the subendocardial Purkinje fiber network through which the pacemaking action

**Fig. 2.** Developmental changes of ventricular activation sequence and the expression of Cx40 and ECE1. (A-C) Isochronal maps of action potential propagation recording on the dorsal ventricular surface of looping, and septating stage hearts. The first activation site is indicated in red. Developmental stages and time resolution of each frame are also indicated. (D) Topological shift of ventricular activation sequences from the immature to the mature pattern during embryogenesis is presented as % of hearts exhibiting the apex-to-base pattern. More than 12 hearts examined at each time point. (E-G) Whole-mount in situ hybridization for Cx40 mRNAs (positive signals indicated by white arrows) in hearts cut to half frontally at stages indicated. Insets are controls stained with sense probes. (H) A transverse section of E8.5 heart stained for Cx40. (I) High power view of the boxed area in H. (J-L) As in E-G but for ECE1 mRNAs. (M) As in H but for ECE1. (N) High power view of the boxed area in M. at, atrium; avj, atrioventricular junction; ec, endocardium; ivs, interventricular septum; lv, left ventricle; ot, outflow tract; rv, right ventricle; tb, trabeculum.



potentials propagate toward the ventricular apex and are transmitted to myocytes in the mature hearts. Histological sections of these stained hearts revealed that subendocardial Purkinje fiber differentiation was restricted to these ridges of trabeculae closest to the ventricular lumen (i.e. the tips of sectioned trabeculae, Fig. 2H,I) but not those embedded in the compact myocardium.

The endocardial expression of ECE1, a key molecular component for defining an induction site of ET-dependent Purkinje fiber differentiation (Takebayashi-Suzuki et al., 2000), was detectable in the outer curvature of the presumptive ventricle (Fig. 2J) where action potentials propagated faster (Fig. 2A) and higher levels of Cx40 expression were detected (Fig. 2E). The outer curvature of the presumptive ventricle has also been thought to be exposed to a higher shear stress and/or stretch than the inner curvature (Thompson et al., 1995; Hogers et al., 1995; Moorman et al., 1998). Thus, the observed ECE1 expression pattern at heart looping stages is consistent with the idea of a hemodynamic and/or biomechanical force-dependent regulation of ECE1 expression.

By E5-7, the endocardial expression of ECE1 was detected along developing trabeculae (Fig. 2K), as seen in the Cx40 expression pattern (Fig. 2F). Higher levels of hybridization signals were detected in developing valve leaflets, as previously reported (Takebayashi-Suzuki et al., 2000). Strong signals were also seen at the crest of the interventricular septum, a prospective site of bundle branch differentiation (Moorman et al., 1998; Cheng et al., 1999). However, again there was no continuous staining for ECE1 from the base to the apex of the ventricle at this stage, which agrees with the optical mapping data that showed the immature lateral

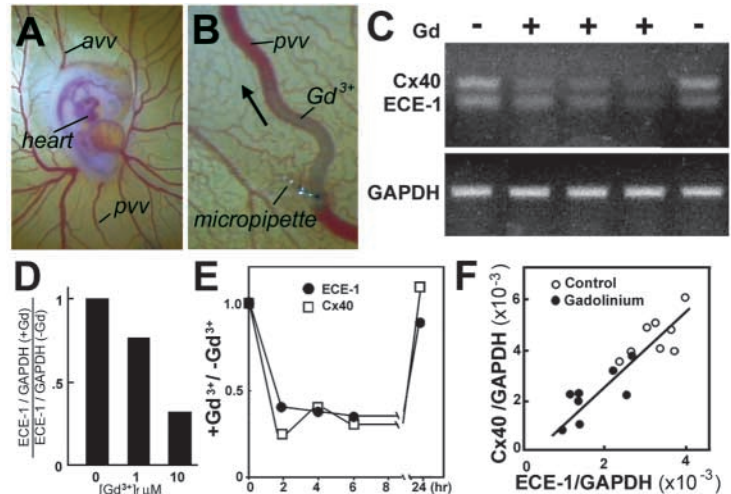
activation sequence at this developmental stage. As the activation sequence shifted from the immature pattern to the mature apex-to-base pattern (Fig. 2C), the endocardial ECE1 expression became more distinct along the longitudinal axis of the ventricle (Fig. 2L), resembling the Cx40 expression pattern.

In histological sections of these stained hearts, higher levels of ECE1 signals were detected in endocardial cells covering the crests of trabecular ridges (Fig. 2M,N) in close proximity to sites of the differentiation of Cx40-expressing Purkinje fibers were identified (Fig. 2G-I). By contrast, the hybridization signals were significantly weaker in endocardial cells invaginating deeply into the myocardium. The spatially regulated progression of the ECE1 expression pattern is consistent with its function in defining the sites of Purkinje fiber differentiation in the embryonic heart (Takebayashi-Suzuki et al., 2000). The data are also consistent with the idea that ECE1 expression in the embryonic heart may be regulated by blood flow-induced shear-stress and/or a pressure-dependent stretch.

### Downregulation of ECE1 and delayed conduction patterning by gadolinium

If these biomechanical forces serve as a regulatory component of the endocardial expression of ECE1 in the embryonic heart, the suppression of mechanosensors, such as a gadolinium-inhibited stretch-activated cation channel  $I_{SA}$  (Lansman et al., 1987; Yang and Sachs, 1989; Naruse et al., 1998), should result in the downregulation of endocardial ECE1 expression. In addition, changes in the expression of this essential enzyme for active ET production should lead to an alteration of ET-dependent induction of Purkinje fiber differentiation. To test

**Fig. 3.**  $I_{SA}$  channel antagonist-induced downregulation of ECE1 and Cx40 expression levels in vivo. (A) In ovo image of E3.5 embryo during injection through the anterior or posterior vitelline veins (avv and pvv). (B) High-power view of the microinjection site into the posterior vitelline vein. Arrow indicates direction of blood flow carrying the injected gadolinium ( $Gd^{3+}$ )-containing Tyrodes's saline visualized by Trypan Blue. (C) Gel displaying RT-PCR amplicons of GAPDH, ECE1 and Cx40 from control (-) and gadolinium (+)-injected E4 ventricles. (D) Dose response of ECE1 expression to gadolinium injection. The data of three independent experiments were averaged and normalized by those of control.  $[Gd^{3+}]_f$  indicates the estimated final concentration of injected gadolinium (3  $\mu$ l of 0 mM, 10 mM and 100 mM), using average wet weight of embryos as the total volume. (E) Time course of ECE1 and Cx40 expression levels following injection of 3  $\mu$ l of 10 mM gadolinium-containing Tyrode's saline at E7.5. The data are presented as the ratio of expression levels in gadolinium-injected hearts to those in control saline-injected hearts and normalized to GAPDH. (F) Relative expression levels of ECE1 and Cx40 to GAPDH in individual ventricles of E8 hearts injected with control (open circle) and 10 mM gadolinium-containing (filled circle) Tyrode's saline at E7.5. Line corresponds to best fit analysis of data points.



these possibilities in ovo, gadolinium ions were introduced directly into the circulating blood flow at various stages during Purkinje fiber development (Fig. 3A,B). The levels of ECE1 and Cx40 transcripts in the ventricle of the resulting hearts were then examined using RT-PCR analysis (Fig. 3C-F).

Under our RT-PCR conditions, both ECE1 and Cx40 amplicons were detectable after 37 cycles of PCR (Fig. 3C), while PCR signals of a house keeping gene product GAPDH were detectable by 29 cycles. Importantly, no significant difference in the band intensity of GAPDH amplicons was detected between the buffer-injected control group and the gadolinium-injected experimental group. As there was no detectable influence of gadolinium ions on the GAPDH expression, the level of GAPDH expression was used to normalize the expression levels of ECE1 and Cx40. By contrast, the lower intensities of ECE1 amplicons were evident repeatedly in the gadolinium-injected hearts (Fig. 3C). Parallel to the decreased ECE1 expression, the significant downregulation of Cx40 expression was consistently detected in gadolinium-injected hearts compared with control. The expression of vascular endothelial growth factor (VEGF), a known shear stress/stretch-response gene (Li et al., 1997), was also reduced by gadolinium injection (not shown). These results show while gene expression can be regulated by gadolinium ions, others are unaffected.

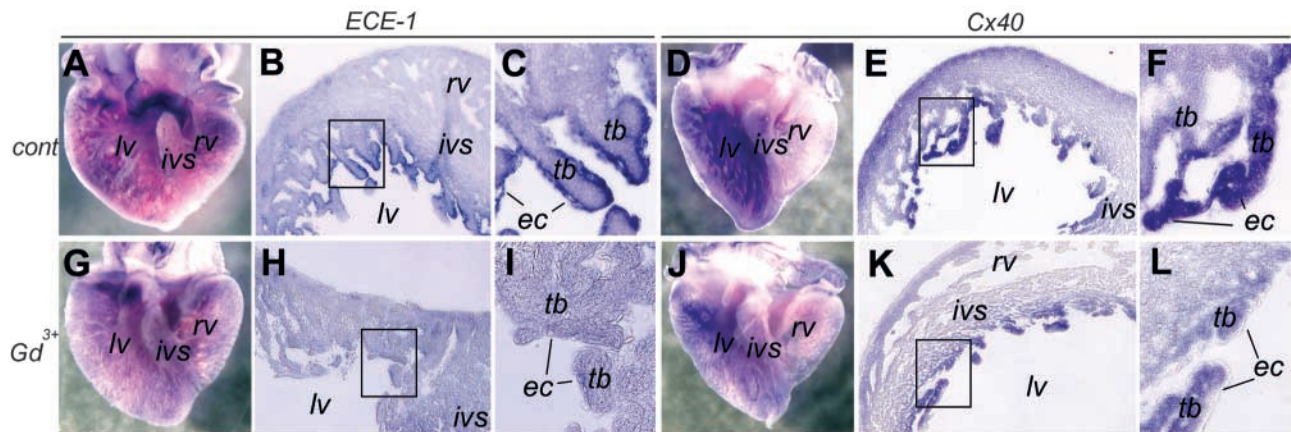
Quantification of the RT-PCR data revealed that gadolinium ions downregulated ECE1 expression in a dose-dependent manner (Fig. 3D). The gadolinium-induced downregulation of ECE1 expression was detectable within 2 hours of injection and the decreased expression levels were sustained for several hours (Fig. 3E). Importantly, however, ventricular ECE1 expression in gadolinium-injected hearts returned to the same level as control hearts 24 hours after injection, indicating that the inhibitory effect of gadolinium on ECE1 expression was reversible. The response of Cx40 expression to gadolinium ions followed the same time course and same levels of downregulation as that of ECE1 (Fig. 3E).

We have previously shown that the Purkinje fiber marker Cx40 is the earliest known responsive gene in ET-induced

conversion from myocytes into Purkinje fibers, both in vivo (Takebayashi-Suzuki et al., 2000; Takebayashi-Suzuki et al., 2001; Kanzawa et al., 2002) and in vitro (Gourdie et al., 1998). Therefore, the expression levels of Cx40 were compared with those of ECE1, the enzyme essential for production of active ET-peptides, in individual hearts (Fig. 3F). Consistent with the known dependency of Cx40 on ET signals in the heart, a two-dimensional plot of the expression levels of Cx40 and ECE1 in eight control hearts and eight gadolinium-injected hearts revealed that the expression level of Cx40 in the ventricle was positively correlated with those of the ECE1, both in control and gadolinium injected groups.

The above RT-PCR analysis clearly demonstrated a gadolinium-induced downregulation of both ECE1 and Cx40 expression in the ventricles. However, the data did not provide any spatial information about the specific sites, if any, affected by gadolinium within the ventricle. To address this issue, in situ hybridization analysis was used to visualize the expression patterns of ECE1 and Cx40 in the heart (Fig. 4). In control hearts, ECE1 signals were readily detected in endocardial cells overlying trabeculae (Fig. 4A-C) that differentiated into Cx40-positive Purkinje fibers (Fig. 4D-F) at the inner most ventricular myocardium. Although gadolinium-injected hearts showed no detectable alteration in heart morphogenesis, such as the chamber size and interventricular septum development (Fig. 4G-L), they displayed lower hybridization signals of ECE1 in the endocardium (Fig. 4G-I) consistent with RT-PCR data. These data demonstrate that endocardial cells are a target site of the gadolinium-induced inhibition of ECE1 expression in the ventricle. Concomitant with a decrease in ECE1 expression in the endocardium, Cx40 signals were diminished in the underlying trabeculae, particularly at the apical regions (Fig. 4J-L). The data suggest that gadolinium injection also induced a significant disruption and/or delay of Purkinje fiber differentiation and patterning.

This conclusion was further verified by changes in the ventricular activation patterns in gadolinium-injected hearts between E7.5 and E8.5 (Fig. 5), during which a topological shift of the ventricular activation sequence from an immature,

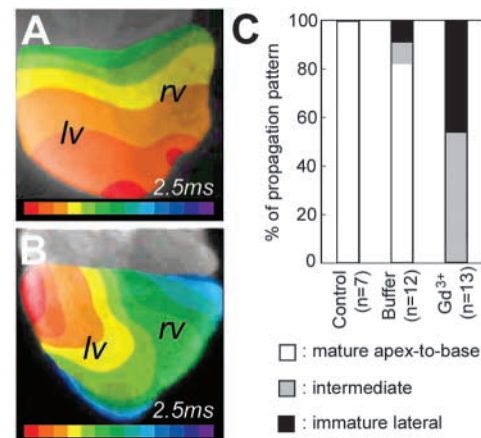


**Fig. 4.** In situ hybridization for ECE1 (A-C,G-I) and Cx40 (E-F,J-L) in E8-8.5 control (A-F) and gadolinium-injected (G-L) hearts. High power view of boxed areas in transverse sections (B,E,H,K) of the ventricle of whole-mount stained hearts are presented in C,F,I,L, respectively. at, atrium; avj, atrioventricular junction; ec, endocardium; ivs, interventricular septum; lv, left ventricle; ot, outflow tract; rv, right ventricle; tb, trabeculum.

lateral pattern to a mature, apex-to-base pattern normally takes place (Fig. 1). In this set of experiments, all of seven uninjected hearts exhibited a mature apex-to-base pattern. Similarly, 10 out of 12 Tyrode's saline-injected control hearts showed a mature propagation pattern (Fig. 5A,C). One Tyrode's saline-injected heart displayed an intermediate pattern in which the activation initiated at the right ventricular apex and conducted up to the right ventricular base, then down the left ventricular apex. Another Tyrode's saline-injected heart exhibited an immature pattern. In striking contrast, none of the gadolinium-injected displayed a mature conduction pattern. Of 13 gadolinium-treated hearts, seven showed an intermediate activation pattern and six exhibited a typical immature lateral activation sequence (Fig. 5B,C). The differences in the ventricular activation sequences between the two experimental groups suggested that gadolinium-injected hearts failed to develop a mature pattern of the ventricular conduction network during this developmental window. Taken together with the RT-PCR and in situ hybridization data, it was concluded that an  $I_{SA}$  channel antagonist, gadolinium, was capable of suppressing both endocardial ECE1 expression and patterning of Purkinje fiber differentiation.

#### Enhanced ECE1 expression and conduction pathway maturation by pressure overloading

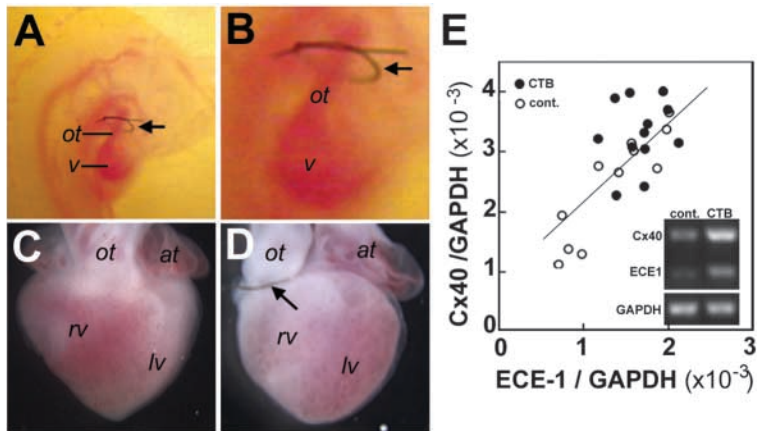
The above response of the endocardial ECE1 expression to an antagonist for  $I_{SA}$  channels suggested that biomechanical force, such as blood flow and/or pressure-induced stress and stretch, may regulate the ECE1 expression in the embryonic heart. If this was the case, an increase of biomechanical inputs to the embryonic heart should activate endocardial ECE1 expression and should promote Purkinje fiber differentiation and patterning. To test this possibility, a pressure-overload was introduced to the ventricle by an established conotruncal banding (CTB) method carried out at E3.5 (Fig. 6A,B) (Sedmera et al., 1999; Tobita et al., 2002). In our initial test of survival, 36 of a total of 58 CTB embryos (62%) survived until E7.5-8, while 10 of 15 (67%) of sham control embryos and 10 of 12 (83%) unoperated control embryos survived over a similar period. The expression levels of ECE1 and Cx40 in the



**Fig. 5.** Delayed patterning of ventricular activation sequences by gadolinium-injection at E7.5. (A) Isochronal map of control E8 heart with the apex-to-base activation pattern. (B) As in A but gadolinium-injected heart still exhibiting an immature lateral activation pattern. (C) Proportion of hearts exhibiting mature (white), intermediate (see text) (gray) and immature (black) activation patterns following no injection (control), buffer and gadolinium injection. Chi Square test between buffer- and gadolinium-injected groups gave a significant  $P$  value of 0.00012, while it found an insignificant  $P$  value of 0.521 between uninjected (control) and buffer-injected hearts. n, number of hearts examined. at, atrium; avj, atrioventricular junction; ec, endocardium; ivs, interventricular septum; lv, left ventricle; ot, outflow tract; rv, right ventricle; tb, trabeculum.

resulting hearts were examined by RT-PCR and in situ hybridization analyses. Ventricular activation sequences were analyzed by optical mapping as above.

By E6-7, the CTB hearts exhibited a more rounded ventricular apex and right-shifted position of the outflow tract/great vessels than control hearts (Fig. 6C,D), both of which were characteristics of pressure-overloaded embryonic hearts as previously described (Sedmera et al., 1999; Tobita et al., 2002). The expression levels of ventricular ECE1 and Cx40 were examined by RT-PCR in the ventricle of individual CTB-



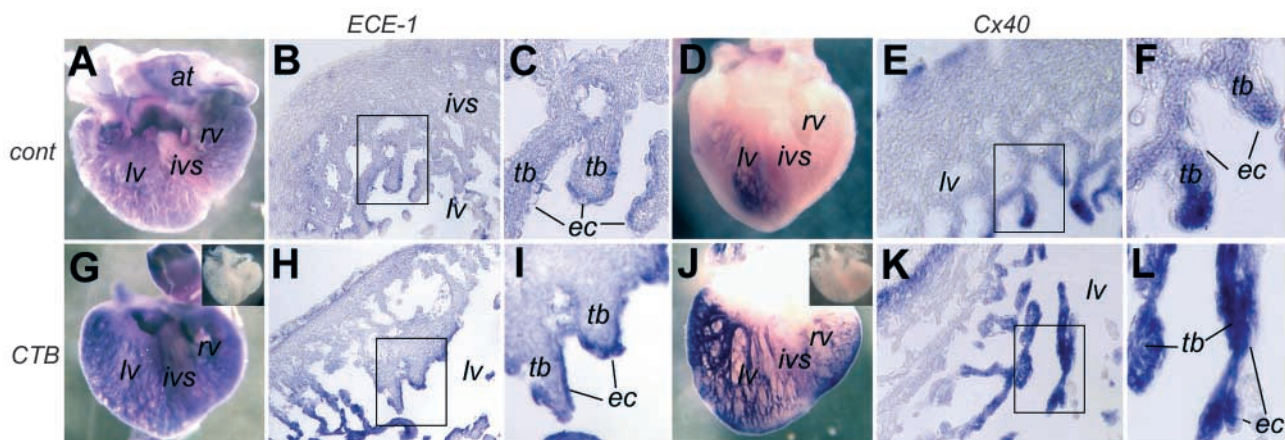
**Fig. 6.** Increased levels of ECE1 and Cx40 expression by CTB-induced pressure overloading between E3.5 and E6-6.5. (A) Video-captured image of an E3.5 embryo just after CTB (arrow, suture). (B) High power view of the heart region in A, showing a banding of the outflow tract without constriction at this stage. (C) E6.5 sham-operated heart. (D) E6.5 CTB (arrow) heart. (E) Relative expression levels of ECE1 and Cx40 normalized to GAPDH in individual ventricles of E6-6.5 control (white circle) and CTB (black circle) hearts. Line corresponds to best fit analysis of data points. Inset is an example of RT-PCR amplicons of GAPDH, ECE1 and Cx40 from control and CTB ventricles.

hearts displaying these morphological characteristics (Fig. 5E). By contrast, an increase in ECE1 expression was detected in CTB-hearts. Again, expression levels of Cx40 showed a positive correlation with those of ECE1 (Fig. 6E). Thus, CTB-induced pressure overload of the ventricle resulted in upregulation of ECE1 and Cx40 expression. The expression of VEGF known to be shear-stress/stretch responsive was also increased in CTB hearts (not shown).

Whole-mount in situ hybridization analysis was then performed to examine whether the CTB-induced increase in expression levels detected by RT-PCR reflected changes in endocardial ECE1 expression and subendocardial Purkinje fiber differentiation (Fig. 7). In control hearts, weak ECE1 signals were detected in endocardial cells overlaying trabeculae (Fig. 7A-C) that differentiated into Cx40-positive putative subendocardial Purkinje fibers (Fig. 7D-F). By contrast, CTB hearts displayed higher hybridization signals of endocardial ECE1 (Fig. 7G-I). Although not quantified, a significant expansion of ECE1-positive endocardial cells within the ventricle was apparent visually. The data demonstrate that endocardial cells are a target site of the CTB-induced upregulation of ECE1 expression in the ventricle. Associated with the upregulated expression of endocardial ECE1, significantly higher levels of Cx40 signals were readily

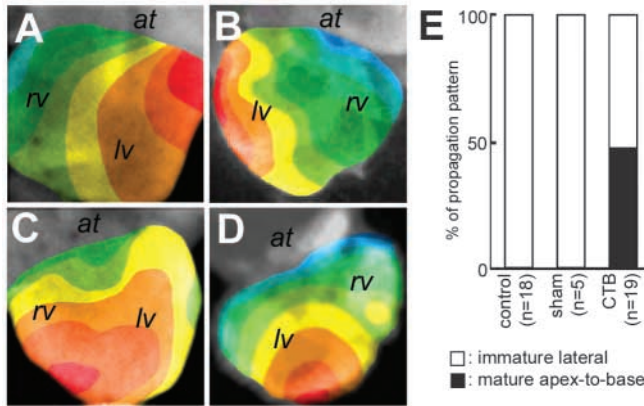
detected in developing subendocardial Purkinje fibers (Fig. 7J-L). Furthermore, it was evident that many Cx40-positive Purkinje fibers ran more along the longitudinal axis than the horizontal axis of the ventricle of CTB-hearts (Fig. 7J). This pattern was not seen in control hearts (Fig. 7D) at this developmental stage. These data show that pressure overload by CTB induced more endocardial cells to express ECE1 at higher levels, as well as a higher density and more anisotropic patterning of putative subendocardial Purkinje fiber differentiation. The experimentally induced changes in Cx40 expression also suggested that a precocious patterning of the Purkinje fiber network may be involved in the recently reported precocious induction of a mature ventricular activation sequence in CTB hearts (Reckova et al., 2003).

To further confirm this possibility, the ventricular activation sequences were compared between control and CTB hearts at E6-E6.5 (Fig. 8), just before normal conversion from an immature lateral propagation pattern to a mature apex-to-base pattern (Fig. 1D). All hearts from unoperated and sham-operated groups initiated the activation at the left ventricular base and conducted impulses laterally through both left and right ventricles (Fig. 8A,B,E), exhibiting a typical immature pattern of ventricular activation sequences. However, the same immature pattern was seen only in 10 out of 19 CTB-hearts. The remaining nine CTB-hearts initiated activation at the apex of the ventricle and propagated impulses toward the base along the longitudinal ventricular axis (Fig. 8C-E), displaying a typical mature activation pattern. Thus, a significant portion of



**Fig. 7.** In situ hybridization for ECE1 (A-C, G-I) and Cx40 (E-F, J-L) in E6-6.5 control (A-F) and CTB (G-L) hearts. High-power view of boxed areas in transverse sections (B, E, H, K) of the ventricle of whole-mount stained hearts are presented in C, F, I, L, respectively. at, atrium; avj, atrioventricular junction; ec, endocardium; ivs, interventricular septum; lv, left ventricle; ot, outflow tract; rv, right ventricle; tb, trabeculum.





**Fig. 8.** Precocious patterning of ventricular activation sequences by ventricular pressure overload (A) Isochronal map of control E6-6.5 heart with an immature lateral activation pattern across the dorsal surface of the ventricle. (B) As in A but the ventral view. (C) As in A but CTB-heart already exhibiting the apex-to-base activation pattern. (D) As in C but the ventral view with a mature pattern. (E) Proportion of hearts exhibiting immature (nonapex-to-base, open bar) and mature (apex-to-base, filled bar) activation patterns following no operation (control), sham operation and CTB. at, atrium; avj, atrioventricular junction; ec, endocardium; ivs, interventricular septum; lv, left ventricle; ot, outflow tract; rv, right ventricle; tb, trabeculum.

CTB-hearts precociously developed a mature pattern of ventricular activation sequences. Taken together with the RT-PCR and in situ hybridization data, it was concluded that a pressure overload to the ventricle is capable of enhancing endocardial ECE1 expression and its downstream Purkinje fiber differentiation. Furthermore, enhanced Purkinje fiber differentiation is capable of the precociously inducing establishment of the mature apex-to-base activation sequence.

The *in ovo* data presented above show that the endocardial ECE1 expression can be regulated in the embryo by pharmacologically antagonizing  $I_{SA}$  channels with gadolinium and by mechanically increased ventricular blood pressure. The data also show that patterning of Purkinje fiber differentiation illustrated by Cx40 expression and electrical activation sequences, follows the changes in endocardial ECE1 expression. These results strongly support the hypothesis that a stretch- and pressure-sensitive mechanism is a regulatory component of endocardial ECE1 expression and its downstream patterning of subendocardial Purkinje fiber differentiation.

## Discussion

The correct patterning of the Purkinje fiber network is crucial for establishing a rhythmic heartbeat. The data from our previous and present studies are consistent with the model whereby a biomechanical force(s) generated by heartbeat and/or blood flow triggers an  $I_{SA}$  channel- and pressure-sensitive mechanism(s), inducing an endothelial cell subpopulation to express ECE1 in the embryonic heart. Only endothelial cells expressing ECE1 can produce active ET peptide, thereby restricting the ET-induced Purkinje fiber recruitment from working myocytes within the embryonic heart.

Although our *in vivo* studies have demonstrated that endocardial ECE1 expression in the embryonic heart can be upregulated by pressure overload, it has been shown in culture that laminar flow-induced shear stress induces vascular endothelial cells to slightly downregulate ECE1 expression, as well as the expression of preproET and production of mature ET (Harrison et al., 1998; Masatsugu et al., 1998; Morawietz et al., 2000). The reason for this contradictory response of endothelial cells seen between *in vivo* and *in vitro* experiments is currently uncertain. One possibility is that cardiac endothelial cells have a distinct responsiveness to hemodynamic loads from other vascular endothelial cells. Alternatively, there might be a mechanism by which endothelial cells distinguish different types of hemodynamic loads. Indeed, it has been shown that preproET expression and production in cultured endothelial cells can be regulated differently by shear stress and stretch (Yoshizumi et al., 1989; Malek and Izumo, 1992; Wang et al., 1993; Macarthur et al., 1994; Zhu et al., 1997; Morawietz et al., 2000; Garcia-Cardena et al., 2001; Chen et al., 2001). In fact, ET secretion by endothelial cells is increased by a combination of oscillatory shear stress and increased blood pressure (Ziegler et al., 1998; Markos et al., 2002), which are similar to hemodynamic loads induced by CTB in the present study. The data are consistent with the normal expression pattern of ECE1 that occurs in endothelial cells of higher tension endocardium and coronary arteries but not in lower tension veins and capillaries (Takebayashi-Suzuki et al., 2000).

Our RT-PCR data have shown that levels of ventricular ECE1 expression are downregulated by gadolinium and upregulated by CTB. In situ hybridization analysis of these hearts has detected endocardial endothelial cells as the main cell type responsible for the induced changes of ECE1 expression levels in the ventricle. However, it is unlikely that only endocardial cells are affected by the  $I_{SA}$  channel antagonist or pressure overload. Indeed, the same antagonist has been shown to block  $I_{SA}$  channels of chick heart muscle (Hu and Sachs, 1996). Furthermore, mechanical stimuli have long been known to alter both the physiological characteristics (Bainbridge, 1915; Rajala et al., 1976; Rajala et al., 1977; Lab, 1980; Ruknudin et al., 1993) and gene expression patterns (reviewed by Brutsaert, 2003) of the heart muscle in both whole hearts and isolated tissue. In addition, shear stress responsive elements (SSRE) have been identified within promoter regions of the ECE1 gene (Orzechowski et al., 1997). Nevertheless, no significant alteration of ECE1 expression was detected in myocytes in hearts that are exposed to the  $I_{SA}$  channel inhibitor or CTB-induced pressure overload. Although the reason for this cell type-specific response is currently unknown, the data obtained in this study provide the first *in vivo* evidence for a higher responsiveness of ECE1 expression to biomechanical inputs in endocardial endothelial cells than myocytes of the embryonic heart.

Our data have shown that Cx40, a Purkinje fiber marker gene, is expressed in trabecular myocytes that are adjacent to the ECE1 positive endocardial endothelial cells. The data have also demonstrated that the gadolinium- and CTB-induced changes in expression levels and patterns of Cx40 occur concurrently with those of ECE1. The data suggest a tight link between the trabecular Cx40 expression with the endocardial ECE1 expression. However, expression of other components in

the ET signaling cascade, such as preproET and ET receptors, may also change in these hearts and may contribute to hemodynamic-dependent patterning of Purkinje fibers. Furthermore, our survey of the database has identified several putative SSRE promoter elements in the Cx40 genes (data accession numbers: rat Cx40, AF025767.1; mouse Cx40, AF023131.1; and human Cx40, AF246295). Therefore, there is the possibility that Cx40 expression is directly regulated by biomechanical forces, independently from ECE1 expression in adjacent endocardial cells. Although this possibility cannot be ruled out, this potential mechanism does not explain the absence of any detectable biomechanical force-dependent response of Cx40 expression in other myocardial regions where myocytes continue a contraction-relaxation cycle. Indeed, the expression of Cx40 by intramural Purkinje fibers adjacent coronary arteries (Gourdie et al., 1993; Gourdie et al., 1995), but not in adjacent working myocytes presents a similar problem, as these cells are likely to be subject to similar levels of wall stress. At the time of their differentiation, periartrial Purkinje fibers are nonetheless juxtaposed to coronary vascular tissues expressing high levels of ECE1 (Takebayashi-Suzuki et al., 2000). In addition, we have previously demonstrated that viral co-expression of ECE1 with the preproET in the embryonic myocardium gives rise to an ectopic conversion of myocytes into Purkinje fibers (Takebayashi-Suzuki et al., 2000). Furthermore, Cx40 is an earliest known responsive gene in this ET-induced Purkinje fiber differentiation (Gourdie et al., 1998; Takebayashi-Suzuki et al., 2000; Takebayashi-Suzuki et al., 2001; Kanzawa et al., 2002). Taken together, it is equally possible that the gadolinium- and CTB-induced changes of Cx40 expression result from an altered production of inductive ET-signals by endocardial ECE1.

To date, ET is the only paracrine signal that has been experimentally demonstrated to induce myocytes to differentiate into Purkinje fibers in the embryonic chick heart (reviewed by Pennisi et al., 2002). In the embryonic mouse heart, however, a potential involvement of other paracrine interactions, such as neuregulin and its receptors, ErbB2 and ErbB4, between endocardial cells and myocytes has also been suggested (Moorman et al., 1998; Rentschler et al., 2002). Neuregulin is secreted by the endocardium, while ErbB2 and ErbB4 are expressed in the myocardium. Conduction disturbances in mice mutant for these genes have been suggested to be a result of insufficient contractile capacity (Moorman et al., 1998). Like ET, neuregulin can induce murine myocytes to upregulate some conduction cell markers, including atrial natriuretic factor and skeletal muscle protein (Zhao et al., 1998). However, it is not known whether Purkinje fiber specific-gene programs observed in the avian embryo, such as the upregulation of Cx40 and downregulation of MyBP-C (Takebayashi-Suzuki et al., 2000; Takebayashi-Suzuki et al., 2001), are regulated by this signal in the mouse. It is also unknown whether neuregulin expression is regulated in a biomechanical input-dependent manner. Interestingly, neuregulin expression is a known downstream component of ET-signaling (Zhao et al., 1998). The interactions between these signaling cascades and the molecular mechanisms underlying induction, differentiation and maturation of Purkinje fibers remain to be determined.

Induced changes in the expression patterns of Cx40 in gadolinium-injected and CTB operated hearts demonstrate that

there is significant plasticity in patterning of the Purkinje fiber network. The diminished and enhanced differentiation of the Purkinje fiber network suggested a delay and acceleration of conversion of the ventricular activation sequence from an immature lateral pattern to a mature apex-to-base pattern, respectively. Our optical mapping data are consistent with this idea. However, an alteration of Purkinje fiber differentiation, the most distal component of the ventricular fast conduction system, may not be a sole cause for these changes in ventricular activation sequences seen in these hearts. Indeed, it has recently been reported that development of the bundle branches, the proximal component of the conduction system, is delayed by LAL-induced blood overload and is accelerated by CTB-induced pressure overload (Reckova et al., 2003). Together with the present study, it is likely that changes in hemodynamic loads can influence development of both proximal and distal components of the ventricular fast conduction system.

The importance of hemodynamic forces in cardiovascular development has long been known. The present study specifically demonstrates that endocardial expression of ECE1, the enzyme essential for producing active ET-peptides, can be modulated by stretch-activated channel antagonist and CTB-induced pressure overload. The experimentally induced changes in Cx40 expression and ventricular activation sequences also suggest that biomechanical forces acting on, and created by, the cardiovascular system during embryogenesis, are a crucial regulatory component of Purkinje fiber induction and patterning. These findings would also provide a basis to further elucidate how certain regions of the endothelium produce ECE1.

We thank Drs D. A. Fischman, D. E. Reese and V. Ballard for their valuable comments on this manuscript. Our thanks extend to Ms L. T. Zagreda and Ms A. F. Dizon for their technical assistance. The work is supported in part by grants from the NIH (HL54128, HL62175 and HL67150 to T.M., HD39446 to R.R.G. and RR16434 to D.S.).

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