To: Dr. Jim Fourqurean, Chairperson
Department of Biological Sciences

This Undergraduate Honors Thesis in Biological Sciences, written by Chulhan Kwon, and entitled "CARDIAC NEURAL CREST CELLS AND THEIR ROLE IN MURINE PURKINJE FIBER DEVELOPMENT", is submitted to you in partial fulfillment of the requirements for Undergraduate Honors in Biological Sciences. The Biological Sciences Undergraduate Honors Committee and the candidate's research supervisor(s) have read this thesis. We recommend that it be approved.

Dr. Lidia Kos,
Honors Research Supervisor

Dr. Jennifer Richards, Chairperson,
Undergraduate Honors in Biological Sciences Committee

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This thesis by Chulhan Kwon is approved.

Dr. Jim Fourqurean, Chairperson
Department of Biological Sciences

Department of Biological Sciences
Florida International University

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Department of Biological Sciences

Florida International University

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ABSTRACT OF THE UNDERGRADUATE HONORS THESIS

IN BIOLOGICAL SCIENCES

CARDIAC NEURAL CREST CELLS AND THEIR ROLE IN MURINE PURKINJE FIBER DEVELOPMENT

By

Chulhan Kwon

Florida International University

Miami, Florida

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Major Professor: Dr. Lidia Kos, Associate Professor

The heart beat is regulated by the cardiac conduction system (CCS), a specialized group of cells that transmit electrical impulses around the heart chambers. During development, ventricular CCS cells originate from embryonic cardiomyocytes and not from the neural crest. Nonetheless, discoveries in chick implied that the cardiac neural crest (CNC) cells contribute to proper development of the ventricular CCS. In this report, the Splotch mouse mutant ($Pax3^{Sp}$), in which the CNC cells do not migrate to the heart, was used to investigate whether these cells also affect proper CCS development in mammals. Homozygote mutants ($Pax3^{Sp/Sp}$) are lethal on

Embryonic Day 13 (E13), and can be phenotyped by spina bifida and exencephaly. *Pax3*$^{Sp/+}$ mice were crossed to obtain wild type, *Pax3* $^{Sp/+}$ and *Pax3* $^{Sp/Sp}$ embryos. Comparison of hematoxylin and eosin stained histological sections showed less trabeculation in E12.5 cardiac ventricles of *Pax3*$^{Sp/Sp}$. Furthermore, immunofluorescence analysis with the Purkinje fiber marker Cx40 showed a qualitative difference between wild type and mutant hearts. Quantitative analysis indicated that *Pax3* $^{Sp/Sp}$ ventricles had fewer Cx40 expressing cells, as well as less Cx40 being expressed per cell when compared to wild type ventricles. Immunofluorescence with the H^3 histome mitosis antibody showed fewer proliferating cells in the ventricles of mutant embryos when compared to controls. These results suggest that CNCC affect the morphogenesis of cardiac ventricles and the development of the ventricular CCS by contributing cellular proliferation.

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ACRONYMS

Acronym	Meaning
CNC	Cardiac neural crest
CNCC	Cardiac neural crest cells
Cx40	Connexin-40
ACF	Animal Core Facility
AV bundle	Atroventricular bundle
AV node	Atrioventricular node
BMP	Bone morphogenetic protein
CCS	Cardiac conduction system
E	Embryonic day
ET-1	Endothelin-1
H&E stain	Hematoxylin & Eosin stain
H3MM	Anti-phospho-Histone H3 (Ser10), Mitosis Marker
IACUC	Institutional Animal Care and Use Committee
NCC	Neural crest cells
NRG	Neuregulin
Sp	Splotch
$Pax3^{Sp}$	Homozygous Pax3 mutant
EtOH	Ethanol
PBS	Phosphate-buffered saline
DEPC-PBS	Diethylpyrocarbonate-Phoshpate-buffered saline
BSA	Bovine serum albumin
pH	Potential of Hydrogen
PI	Propidium iodide
MBS	Mixed buffered solution

INTRODUCTION

The heart is mostly formed by the cardiogenic mesoderm, which gives rise to endocardiac endothelial cells, atrial myocytes, and ventricular myocytes. The ventricular myocytes differentiate into Purkinje fibers, a specialized group of cells that propagate electrical impulses through gap junction proteins, such as Connexin 40, 43, and 45 (Thomas et al., 1998; Gilbert, 2003). Another population of cells contributing to heart development is the neural crest cells (NCC). Cardiac NCC (CNCC), located in the caudal region of the cranial neural crest, contribute to the septation of the outflow tract and give rise to smooth muscle cells (Kirby, 1989; Kuratani and Kirby, 1991; Waldo et al., 1996; Waldo et al., 1998); (Conway et al., 1997a; Conway et al., 1997b; Conway et al., 2000; Gilbert, 2003). apart from these well studied functions of the CNCC, recent evidence obtained with chick and zebrafish embryos suggest that they may also participate in the development of the cardiac conduction system (CCS) (Gilbert, 2003). However, little is known about their role in the development of the mammalian CCS. This research investigates the role of CNCC on the development of the murine ventricular CCS.

CNCC and heart development. The significance of cardiac neural crest cells (CNCC) in chick cardiac development has been well-studied (Bockman et al., 1989; Kuratani and Kirby, 1991; Waldo et al., 1996; Waldo et al., 1998; Farrell et al., 1999; Waldo et al., 1999). Following ablation (surgical removal or laser destruction), CNCC did not populate near the pharyngeal arches or migrate to the outflow tract (Waldo et al., 1999). In the absence of CNCC, the chick heart had abnormal myocardial development but a normal endocardium (Waldo et al., 1999). This implied that CNCC played a role in differentiation of early myocardium through maturity.

Genes expressed among the CNCC. A few mouse spontaneous mutants showing abnormalities in the CNCC have been used to addres their potential role in heart development. Patch

mice have alternations in the regulatory regions that control both the platelet-derived growth factors (PDGF)–alpha receptor gene and the kit gene. Heterozygous mice have a white belly spot, while homozygous embryos are lethal. Cardiovascular anomalies include persistent truncus arteriosus (PTA), as well as abnormal aortic arch arteries. It is thought that altered expression of both genes contribute to abnormal development of the cardiovascular system in mammals (Orr-Urtreger et al., 1992; Besmer et al., 1993; Wehrle-Haller et al., 1996; Soriano, 1997).

Another gene expressed among the CNCC is paired box gene 3 (*Pax3*) (Jackson Laboratory, 2006). *Pax3* is a member of the paired family of transcription factors that have two DNA binding sites along with a homeobox (Goulding et al., 1991; Goulding et al., 1993; Jackson, 2006). Homeobox genes are transcription factors that contain a 180 base-paired consensus sequence that codes for their DNA binding domain. This domain then allows a sequence of 60 amino acids known as a homeodomain to be produced (Klug et al., 2006). *Pax3* is expressed in the CNCC and plays an important role in cardiac development (Kirby, 1988; Epstein, 1996; Conway et al., 1997b; Li et al., 1999). Mutations in *Pax3* lead to lack of proliferation and migration of CNCC to the heart. Heterozygous mice for *Pax3* have a white belly spot due to the improper migration of melanocytic precursor cells. White spotting can also be observed on the back, feet and tail. *Pax3* homozygous embryos suffer severe developmental defects (Epstein, 1996; Epstein et al., 2000). These include improper neural tube development, lack of limb muscles, and lethality around embryonic day 13 (E13) (Epstein, 1996; Epstein et al., 2000); (Epstein, 1996; Conway et al., 1997b; Conway et al., 2000; Epstein et al., 2000; Jackson, 2006). Excessive neuronal apoptosis leads to an unclosed neural tube, clinically manifested as exencephaly, spina bifida, or curly tail. The brain in a *Pax3* homozygous embryo suffers from an abnormal increment

in mitosis and a prolonged cell generation time. These cellular abnormalities result in growth of brain tissue out of the unclosed neural tube (Jackson, 2006).

The *Pax3*$^{Sp/Sp}$ mutation is a result of a shortened *Pax3* transcript, which causes the phenoltypes described above. Additionally, these mutants present abnormal migration of CNCC. These CNCC communicate via gap junctions, such as those formed by Connexin 43 (Cx43) as they migrate to the heart ((Farrell et al., 1999; Waldo et al., 1999; Maschhoff and Baldwin, 2000). The *Pax3*$^{Sp/Sp}$ gene expressed by the CNCC allows proliferation of these neural crest precursors, which leads to a sufficient population of CNCC. Specific receptors, such as endothelin-A and endothelin–B receptors, are expressed by the NCC, and specific factors that are secreted by non-neural crest cells in the pharyngeal arches supports the navigation of NCC, as well as differentiation of cardiac cells. The behavior of *Pax3*$^{Sp/Sp}$ CNCC during their proliferation and migration seems to be affected by an intrinsic property, as well as by the extrinsic environmental factor (Conway et al., 1997b; Chan et al., 2004). The lack of *Pax3* expression causes a lack of proliferation of CNCC in the neural tube, which leads to a deficiency of CNCC.

Cardiac Conduction system (CCS) and CNCC. Presence of CNCC appears to contribute to normal trabeculation in the ventricles of a chick heart (Waldo et al., 1999). The role of CNCC in the mammalian ventricular conduction system, however, has not been studied. The cardiac conduction system is composed of pacemaker cells found in the sinoatrial node, atrioventricular node, bundle of His, and left and right branches of Purkinje fibers in the ventricles (Gourdie et al., 1993; Cheng et al., 1999; Takebayashi-Suzuki et al., 2001; Patel and Kos, 2005). The gap junction protein Connexin 40 (Cx40) is found primarily among the Purkinje fibers and has been utilized as a marker for the ventricular conduction system (Sedmera et al., 2003). Purkinje fibers are electric conducting cells differentiated from cardiomyocytes (Cheng et al., 1999). While much

research has investigated the effect of CNCC on outflow tract septation, their involvement in the development of the ventricular CNCC is poorly reported. NCC, along with instructive factors such as Endothelin or Neuregulin, may support a complete differentiation, and maturation of Purkinje fibers in the ventricles in mammals (Poelmann et al., 2004). To clarify the role of CNCC in CCS development, we studied the development of Purkinje fibers in the ventricles of *Pax3*$^{Sp/Sp}$ mice. We investigated the effect of an insufficient population of CNCC on the ventricular CCS. This research reports that CNCC might have an effect on the development of the ventricular CCS.

MATERIALS AND METHODS

Mice. *Pax3*$^{Sp/Sp}$ mice were originally obtained from Jackson Laboratories (Bar Harbor, Maine). Five heterozygous *Pax3*$^{Sp/Sp}$ males were used repeatedly as male parents in crosses. Populations of heterozygous *Pax3*$^{Sp/Sp}$ females were raised by crossing C57BL/6J female mice with the *Pax3*$^{Sp/Sp}$ males. Females were placed in the male cages with unchanged bedding for three days. Two females were then crossed with a *Pax3*$^{Sp/Sp}$ male mouse at 5:30 PM. An embryonic day (E) 0.5 was assigned to the embryos with a vaginal plug at 7:00 AM the following morning. Pregnant females were separated and kept in the Animal Core Facility (ACF) until E12.5 when the females were sacrificed for their embryos. Animal core protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Florida International University.

Embryo Collection and Preparation. All three genotypes of *Pax3*$^{Sp/Sp}$ mutant embryos were obtained from the littermates. Since the homozygous *Pax3*$^{Sp/Sp}$ embryos were differentiable by the presence of spina bifida (sb) and exencephaly (ex) (Fig. 1B), the dissected embryos were separated into *Pax3*$^{Sp/Sp}$ and control embryos (Fig. 1A). The control embryos constitute both wild type and *Pax3*$^{Sp/Sp}$ heterozygotes. All embryos were dissected in Phosphate-Buffered Saline (PBS) at pH 7.4. The embryos were fixed in 4% Paraformaldehyde for 16 hours. They were later treated with 5% sucrose in PBS for 2 hours and in 10% sucrose for 16 hours. The embryos were individually embedded in plastic molds with freezing embedding medium (Triangle Biomedical Sciences, Durham, North Carolina). Frozen embryos were stored in -20°C for two days. Embryos were transversely sectioned at 10 μm in a Leica cryostat CM30505555 (Nussdoch, Gremany). Sections were dried at 37°C for an hour and stored in -80°C for further use.

Hematoxylin & Eosin (H&E) stain. In order to study the structural differences in the atria and ventricles of *Pax3*^{Sp} and those of control slides were stained with H&E. One slide from each of three control and three homozygous embryos were dried in a slide warmer at 37°C for 1 hour. The sections were submerged in Tris Buffered Saline (TBS) at pH 7.4 for 10 minutes. They were then washed with distilled water for 3 minutes. Sections were stained in undiluted Mayer's Hematoxylin for 1 minute, then washed in distilled water for 3 minutes and again rinsed in running tap for 3 minutes. They were counterstained in undiluted eosin for 30 seconds. Sections were dehydrated and cleared by passing through an ethanol series (70% EtOH for 20 seconds, 95% EtOH for 2 minutes, 100% EtOH for 5 minutes, and 100% EtOH for 5 minutes). Sections were mounted with antifade reagent in glycol (Molecular probes, Eugene, Oregon) and a coverslip added. To visualize the phenotypic differences of the heart between the homozygous *Pax3*$^{Sp/Sp}$ and control embryo sections, digital images were taken with a Leica digital camera DC500 (Wetzlar, Germany) mounted on a Leica fluorescence DMRME microscope, Type 301-371-011 (Wetzlar, Germany).

Connexin 40 (Cx40) Immunofluorescence Analyses. One slide with 10 sections was prepared from each of three control and three homozygous embryos and dried on a slide warmer at 37°C for 75 minutes. These slides were washed for 10 minutes three times in Phosphate Buffered Saline (PBS) with 0.1% of diethylpyrocarbonate (DEPC-PBS) at pH 7.36 s. The sections were blocked in 10% Bovine Serum Albumin (BSA) (Fisher Scientific, Fair Lawn, New Jersey) in DEPC-PBS for 1 hour and 15 minutes, then treated for 1 hour and 15 minutes with rabbit anti-Cx40 primary antibody (Chemicon, California) at a final concentration of 1:100 in PBS homogenized with 5% BSA (Fisher Biotech, NJ). Two washes of PBS for 10 minutes followed. The sections were treated with Alexa-488-conjugated goat anti-rabbit secondary antibody (Jackson Im-

munochemical, Bar Harbor, Maine) at 1:200 concentration in PBS with 5% BSA, and then washed twice in PBS for 10 minutes. Nuclei were stained with propidium iodide (PI) (Fisher Scientific, Fair Lawn, New Jersey) at 1:1000 concentration for 1 minute. The slides were briefly rinsed with PBS. Antifade reagent in glycerol (Molecular Probes, Eugene, Oregon) and glass coverslips were applied to the mounted sections..

Sections were analyzed for the total number of ventricular cells, number of Cx40 positive cells, and number of fluorescent dots by taking 100X magnification images and merging them on a large canvas in Adobe Photoshop. The images were obtained from the same Leica instruments utilized during H&E stain analysis. A 250 μm^2 square box was digitally drawn on each right ventricle where the lower edges of the square met the outer edge of myocardial cushion. The total number of cells (PI labeled) and the total number of Cx40 positive cells present within the box were manually counted. Mean values were compared using Student's t-test and considered to be significantly different at $P \leq 0.05$.

Proliferation Assay. H&E, PI and Cx40 stains showed a lower quantity of cells present in the ventricles of $Pax3^{Sp/Sp}$ mutants. In order to investigate the proliferation level of cardiomyocytes and Purkinje fibers within the ventricles of $Pax3^{Sp/Sp}$ and control, anti-rabbit-H3 histone mitosis marker (H3MM) (Upstate, Lake Placid, New York) was used to stain the proliferating cells within the ventricles.

Embryos were transversely sectioned to study the level of proliferation and population of cells in the ventricles of E12.5 control and E12.5 $Pax3^{Sp/Sp}$ mutant embryos. All sections containing ventricles were subjected to H3MM. The slides were blocked in a mixed buffered solution (MBS) of 1% BSA, 0.1% 10x Trition in PBS-DEPC at pH 7.4 for 1 hour. This was followed by primary antibody treatment of 250 µl of 1:100 concentration of H3MM in MBS for 1 hour and

15 minutes. The sections were washed in MBS three times for 15 minutes each, followed by application of 250 μl of 1:200 concentration of secondary goat anti-rabbit fluorescein in MBS for 1 hour. The slides were then washed in MBS three times for 15 minutes each. The nuclei were counterstained with 1:1000 PI for 1 minute. The sections were washed in MBS three times for five minutes each and mounted. The number of H3mm positive cells were qualitatively compared between mutants and control.

RESULTS

Pax3 mutant embryos showed ventricles with altered structures.

The shapes and sizes of the ventricles of E11.5-E13.5 *Pax3*$^{Sp/Sp}$ and control embryos were different. The control embryo ventricles showed a defined thick endocardium cushion surrounded by noticeable interventricular tissues (Fig. 2A), while the *Pax3*$^{Sp/Sp}$ mutant ventricles showed a thinner endocardium cushion in addition to indistinctive separation of left and right ventricles (Fig. 2B). However, variants of *Pax3*$^{Sp/Sp}$ embryos were also observed which showed generally distorted chambers, no distinct ventricles, and enlarged atria compared to the ventricles (data not shown). The ventricles of *Pax3*$^{Sp/Sp}$ mutant embryos (Fig. 2B) were also comparatively smaller than those of control embryos (Fig. 2A). Finally, less trabeculation was observed from *Pax3*$^{Sp/Sp}$ mutant embryo ventricles (Fig. 2B), while the control embryos ventricles had more trabeculation (Fig. 2A).

Cx40 positive cells from the ventricles of Pax3*$^{Sp/Sp}$ *differed from those of control.

The appearance of Purkinje fibers among the ventricles of the control and *Pax3*$^{Sp/Sp}$ embryos differed qualitatively (Fig. 3). While more Cx40 immunofluorescent dots were seen from the control samples, there were fewer dots from the mutant embryo ventricles. PI stain also showed fewer cells present in the ventricles of *Pax3*$^{Sp/Sp}$ embryos as compared to the control embryos. Quantitative analysis (n=3) showed that 38.8% fewer total cells were present in the ventricles of E12.5 *Pax3*$^{Sp/Sp}$ embryos when compared to controls. The ventricles of E12.5 *Pax3*$^{Sp/Sp}$ had 83.6% less Cx40 positive cells than the ventricles of E12.5 control embryos. E12.5 *Pax3*$^{Sp/Sp}$ embryo ventricles had 89.7% fewer Cx40 fluorescent dots were stained than in those of controls. The ratio in the ventricles of total cells to Cx40 positive cells of *Pax3*$^{Sp/Sp}$ embryos was 72.6% less than the ratio of control embryos. However, the two ratios were not statistically significant

($P \leq 0.05$; Student's t-test). The expression levels of Cx40 membrane proteins per individual Cx40 positive cells were not significantly different between the two groups (Fig. 4E). This ratio of Cx40 positive cells' gap junction protein expression level in $Pax3^{Sp/Sp}$ embryos was 41.5% less than that of controls. Among the five comparisons, the ratios of total cells and fluorescent Cx40 per Purkinje fiber were not significantly different.

The population of proliferating cells in the ventricles of $Pax3^{Sp/Sp}$ embryos qualitatively differed from that of control embryos.

The proliferating characteristics of total cells present in the ventricles of both $Pax3^{Sp/Sp}$ and control embryos were different. When proliferation of cells near the ventricle sections of these samples were observed using primary antibody H3MM simultaneously with PI nuclei stain, more proliferating cells on and near the endocardium cushion, as well as a larger population of PI stained cells were observed in control ventricles (Fig. 5A) when compared to those of $Pax3^{Sp/Sp}$ embryos (Fig. 5B).

DISCUSSION

The goal of this project was to analyze the effect of CNCC on the development of murine ventricular CCS. I hypothesized that the murine CNCC exert an important effect on the development of the ventricular CCS based on that evidence that an ablation of CNCC in chick embryos showed defected outflow tract as well as altered ventricular CCS (Waldo et al., 1999). During development, *Pax3* is actively expressed among CNCC (Epstein, 1996; Epstein et al., 2000). Abnormal cardiac development in *Pax3* homozygous murine embryos had been studied (Epstein et al., 2000). Because $Pax3^{Sp/Sp}$ murine embryos showed altered CNCC development due to a point mutation in this transcription factor, these embryos were an excellent tool to study altered cardiac development due to disrupted CNCC. A genetic disruption among CNCC leads to fewer of these cells to migrate to the outflow tract a developing heart, leading to cardiac morphological defects. In addition to cardiac defects, $Pax3^{Sp/Sp}$ embryos had neural tube closure problems, causing excencephaly and spina bifida.

Another gene seems to contribute to the development of the cardiac conduction system, as well as ventricular myocytes in murine embryos. HF-1b is expressed in the development of peripheral conduction system cells (St Amand et al., 2006). This report showed that NCC also express the HF-1b gene, and these cells cause the atrial and atrioventricular conduction system to mature (St Amand et al., 2006). In our study, we noticed a mutant heart with oversized atria and miniature ventricles (Fig. 2B and I). It is possible that genes such as HF-1b may cause dorsal chambers to differentiate and mature, but not the ventral chambers, such as the ventricles. We suspect that due to deficit in CNCC, atrial development is facilitated by a limited population of CNCC to the heart. An experiment on atrial and ventricular development caused by varying mi-

gration distances of CNCC away from the atria and ventricles may show separate yet sequential interactions among atria, ventricles and CNCC.

Deficits in murine cardiac neural crest cells led to alterations of heart morphology in $Pax3^{Sp/Sp}$ homozygous embryos.

Deficits in CNCC led to cardiac ventricular malformations. Less trabeculation was seen in the ventricles of $Pax3^{Sp/Sp}$ compared to those of controls. Fewer muscular ridges led me to question whether the amount of Purkinje fibers, a component of ventricular conduction system, would also be affected. Cx40 stain for Purkinje fibers and propidium iodide for nuclei stain showed that less Cx40 expression was seen in $Pax3^{Sp/Sp}$ ventricles as well as fewer nuclei stained. The latter confirmed that less trabeculation led to fewer cells. However, since Purkinje fibers have been shown to differentiate from ventricular cardiomyocytes, there was a possibility that $Pax3^{Sp/Sp}$ embryos would show less Cx40 expression not only because of fewer ventricular myocytes undergoing differentiation, but also because of the strength of Cx40 expression per individual Purkinje fiber. Comparisons using Student t-test showed there was no significant difference in the proportion of Cx40 over the total cells present. In addition, the strength of Cx40 expression between $Pax3^{Sp/Sp}$ embryos and the controls had no significant difference either.

Fewer proliferating cells among trabeculae, as well as in the regions of the cardiac conduction system in the ventricles of $Pax3^{Sp/Sp}$ embryos, lead to embryonic lethality.

Because these two comparisons were not significantly different, I conclude that the differentiation of Purkinje fibers from ventricular cardiomyoctes in $Pax3^{Sp/Sp}$ was not affected by the CNCC. This implied that CNCC might play a general effect, either causing the apoptosis of the cells in the ventricles or reducing the proliferation level of these ventricular myocytes and therefore having fewer cells to be differentiated into Purkinje fibers. The proliferation assay

showed that there were fewer proliferating cells in the ventricles of $Pax3^{Sp/Sp}$. Fewer proliferating cells meant fewer cells filling the ventricles and forming trabeculae. This result explained the reduced trabeculation seen in H&E stained sections in the ventricles of $Pax3^{Sp/Sp}$.

Conway et al. (1997) reported that persistent truncus arteriosus in chick embryos resulted from defects among CNCC. This study also showed that there was a less contractile force in the heart due to impaired excitation-contraction coupling Conway et al., 1997). Several studies have shown that there was less calcium in ventricular myocytes in chick embryos, which led to embryonic lethality (Conway et al., 1997a; Conway et al., 1997b; Waldo et al., 1999). We hypothesize that decreased cell populations in the ventricles create a weaker calcium currents, which could lead to physiologically abnormal ventricles of $Pax3^{Sp/Sp}$ embryos. A study of calcium levels of the ventricular myocytes of $Pax3^{Sp/Sp}$ embryo might answer the physiological compatibility which could prevent embryonic lethality.

During zebrafish and chick cardiovascular development, fibroblast growth factor 8 is necessary for cardiogenesis as well as inducing myocardiac precursor signals (Farrell et al., 2001; Schneider et al., 2001). It is possible that a sufficient population of CNCC may secrete a mytogenic factor, such as a member of the FGF family of growth factors, so that various genes can be activated for ventricular myocyte development, allowing proliferation and development of a proper ventricular CCS.

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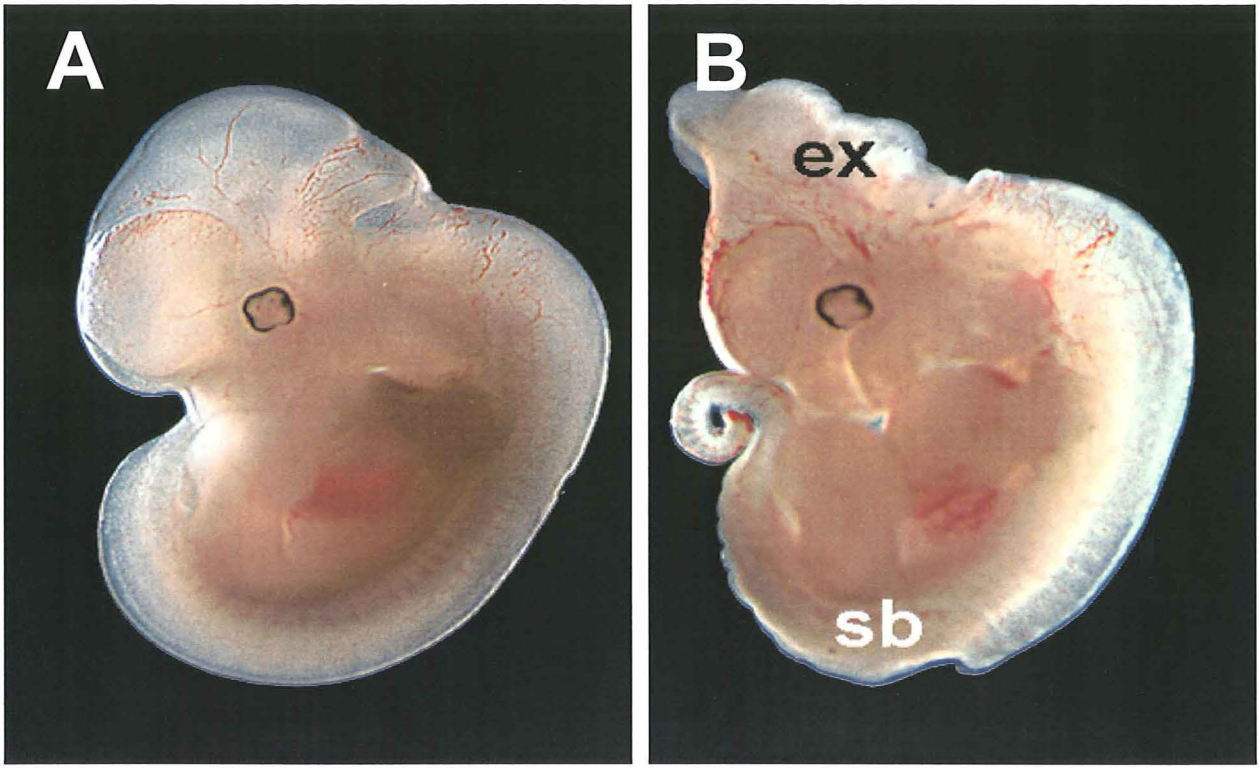

Figure 1. Phenotypic comparison between E11.5 control (A) and *Pax3*$^{Sp/Sp}$ mutant embryo (B). Exencephaly (ex) and spina bifida (sb) are distinguishable characters of these mutant embryos.

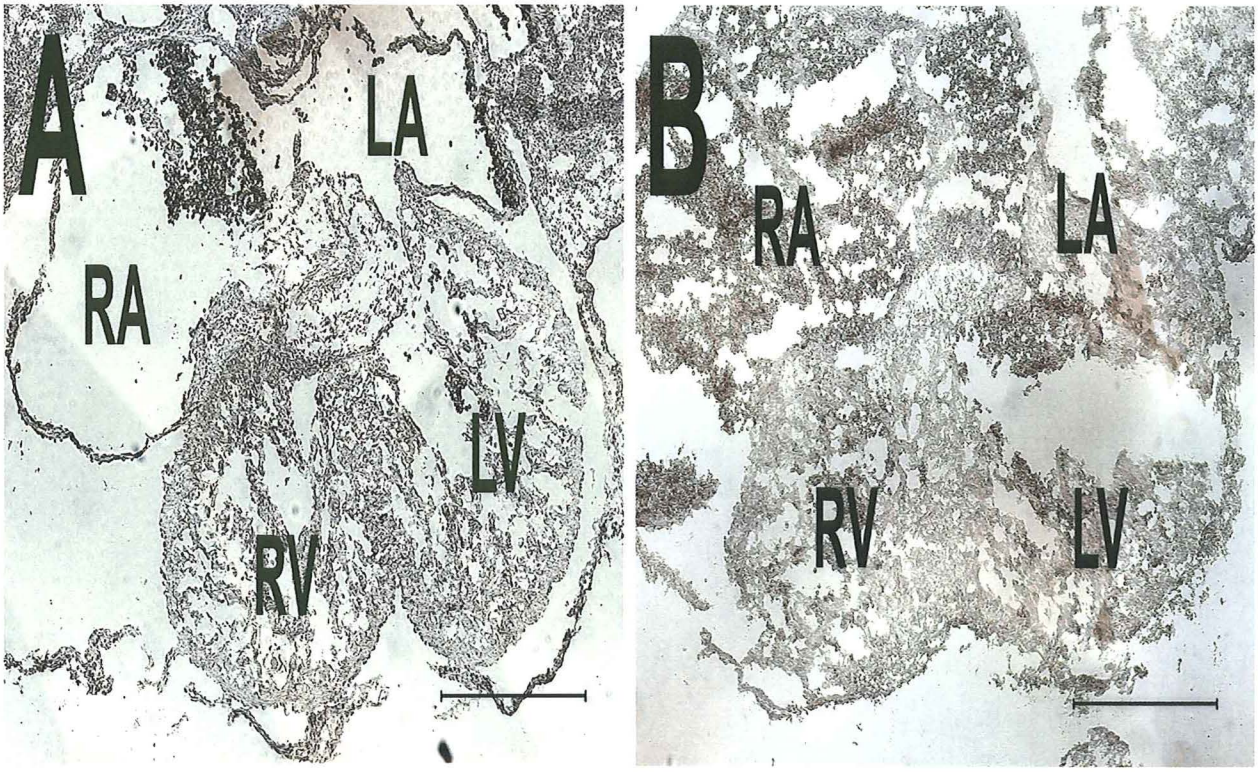

Figure 2. Transverse sections of E12.5 control (A) and *Pax3*$^{Sp/Sp}$ (B) hearts stained with H&E. RV=Right ventricle, LV=Left ventricle, V=Ventricle, RA=Right atrium, RV=Left atrium. Scale bar=500μm.

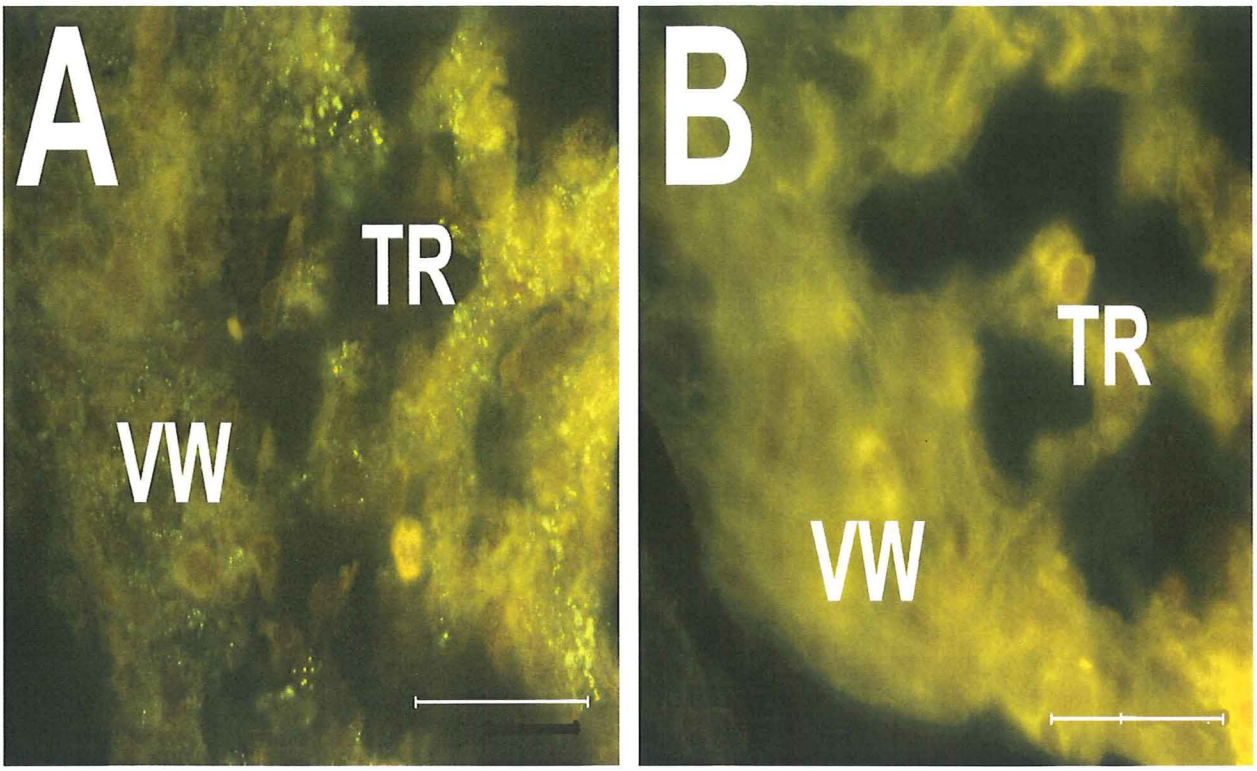

Figure 3. Transverse sections of the ventricles of E12.5 control (A) and *Pax3*$^{Sp/Sp}$ (B). Cx40 stain are seen as bright green dots. Nuclei were counter-stained with propidium iodide (red). TR=Trabeculae VW=Ventricular wall. Scale bar=100μm.

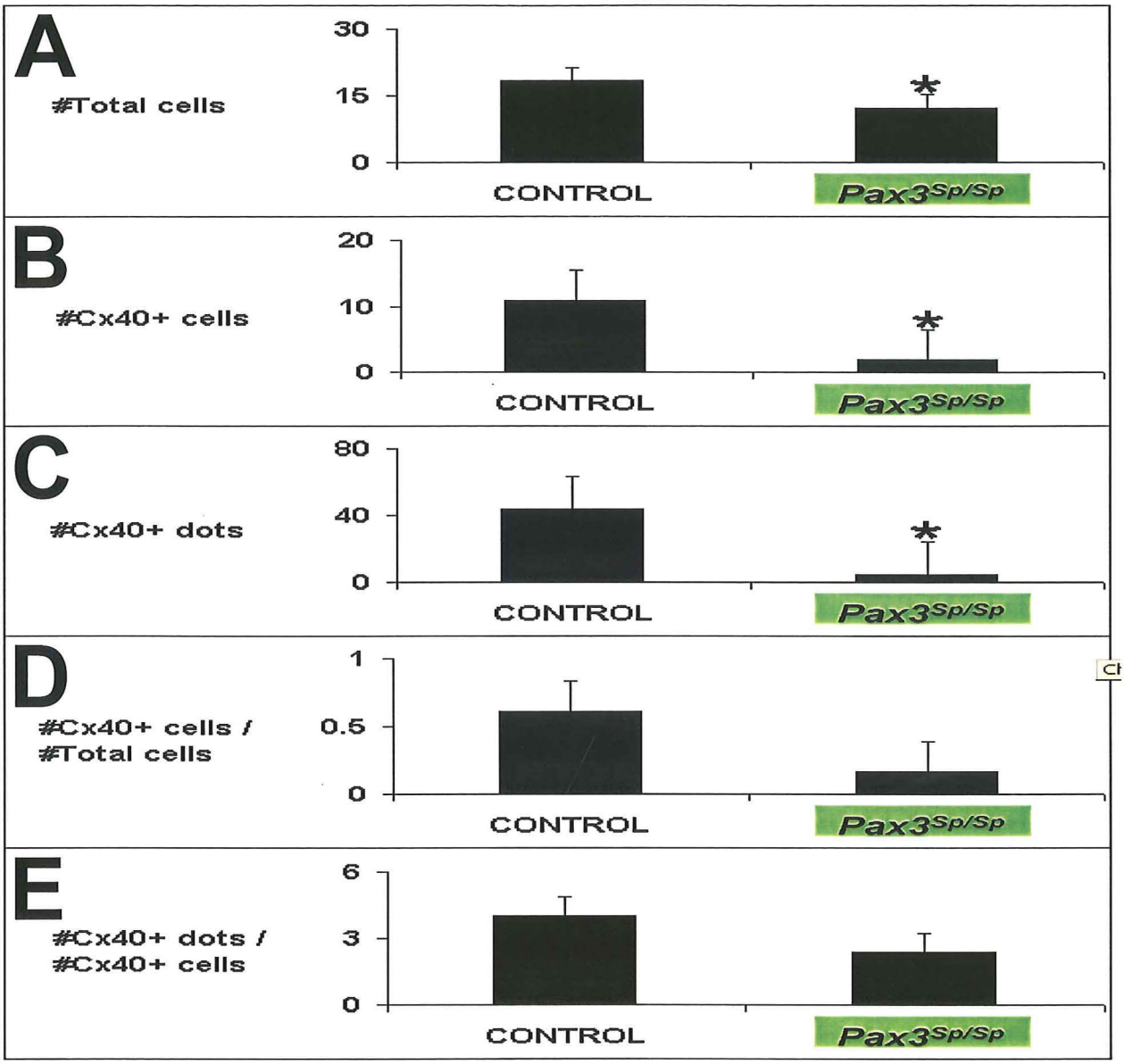

Figure 4. Comparison of the numbers of total cells (A), Cx40 positive cells (B), and Cx40 dots (C) from a 25μm^2 area at the right edge of the right ventricles of E12.5 control and *Pax3*$^{Sp/Sp}$ embryos (n=3). (D) Ratio of Cx40 positive cells over total number of cells showed no significant difference between control and *Pax3*$^{Sp/Sp}$ ventricles. (E) Ratio of Cx40 positive dots over the number of Cx40 positive cells showed no significant difference between control and *Pax3*$^{Sp/Sp}$ ventricles. Bars with asterisks were significantly different at $P \leq 0.05$ in Student's t-test.

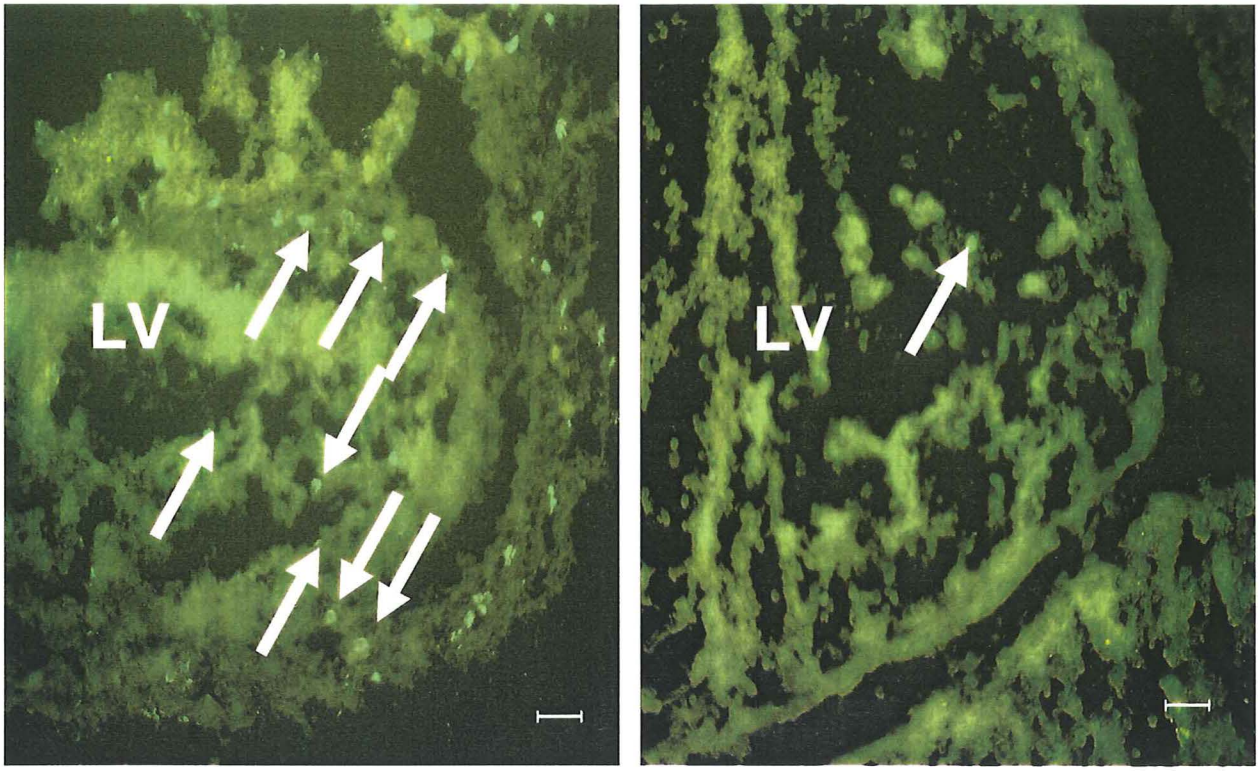

Figure 5. Coronal sections at 20X of E12.5 control (A) and *Pax3*$^{Sp/Sp}$ (B) left ventricles labeled with H3 mitosis antibody (green). Sections from control ventricles had more proliferating cells (arrow) than those from *Pax3*$^{Sp/Sp}$. LV=Left ventricle. Scale bar=100μm.

FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

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