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RESEARCH ARTICLE

Early development of cardiac ventricles: importance of neuregulin 1

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ABSTRACT

Background. Serious ventricular septal defects associated with ventricular hypoplasia and other hyperplasias or total absence of the interventricular septum (IVS) are usually incompatible with embryonic and fetal life. Despite the importance of these cardiac diseases, their causes are not yet known. Using *in vivo* labeling in the chick embryo, the importance of ventricular trabeculation was confirmed in IVS morphogenesis. Using knockout mice and retrovirus in birds, it was determined that lack of function of neuregulin 1 (NRG1) or its ErbB receptors not only causes deficient differentiation of ventricular myocytes and poor formation of trabeculae, but also determines premature death of the embryos. Based on this background, the aim of this study was to determine the actual role of NRG1 in early trabeculogenesis and its importance in proliferation and apoptosis regulation.

Methods. An embryonic chicken heart organ culture system at the age prior to the beginning of the trabeculogenesis process was established. Endogenous activity of NRG1 was inhibited in the organ cultures that were then stimulated with NRG1 at different concentrations. Myocyte proliferation was determined using the proliferating cell nuclear antigen and apoptosis with LysoTracker.

Results. Fetal bovine serum promotes proliferation but negatively impacts trabeculogenesis. Addition of NRG1 at low concentrations and for short periods of incubation does not induce trabeculogenesis. In contrast, average NRG1 concentrations and cultivation periods not exceeding 24 h have a positive effect on the onset of this process. This also promotes myocardial proliferation but avoids apoptosis. Higher concentrations of NRG1 possibly cause a molecular imbalance that favors untidy proliferation but not trabeculogenesis.

Conclusions. Understanding of the role of NRG1 on ventricular trabeculogenesis provides valuable information for the molecular pathways also involved in IVS development. This information is essential for understanding the origin of serious ventricular septal defects.

Key words: ventricular trabeculogenesis, NRG1, early cardiogenesis.

INTRODUCTION

Congenital heart diseases (CHD) involving the interventricular septum (IVS) are called ventricular septal defects. They are among the most common birth defects and have a great impact on pediatric morbidity and mortality.[1] Its frequency is ~0.8/100 live newborns.[1,2] Its complexity depends on the IVS region in which they occur. Septal defects related to trabecular septal regions (middle and apical thirds) can be very simple if they form small communications along this area. They are also extremely serious and complex and often associated with one hypoplastic ventricle and another hyperplastic ventricle. There are even cases in which the IVS is completely absent.[1] These diseases are almost always incompatible with life from the embryonic and fetal stages. However, there is currently no convincing explanation for the origin of these diseases despite the many efforts that have been devoted to clinical and basic research to unravel this mystery. In the field of basic research, the issue has been addressed by studying trabecular development and IVS because it is known that birth defects are formed during embryogenesis. Thus, it

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is known that in the chicken embryo trabecular primordia begin to form soon after completion of the process of torsion and looping of the heart, i.e., stages 16-17HH (Hamburger and Hamilton).[3,4] The first sign is the appearance of small groups of myocytes separated by narrow, apparently empty spaces. The size and length of the myocyte groups then increase and are projected into the ventricular cavity. They remain covered by endocardium on the distal surface in direct contact with the ventricular cavity, giving rise to some incipient trabecular outlines. At the same time, they lengthen and the spaces between them begin to be covered by endocardium. As the trabecular primordia elongate, they branch off to form increasingly differentiated trabeculae.[5] With reference to the development of IVS, Contreras et al. using *in vivo* labeling confirmed the importance of the trabeculae in this process and reported that as the trabecular primordia elongate and branch, they gradually adhere to a central trabeculae to form the primitive IVS, which remodels, increases in length and is reached by the cushions of the atrioventricular canal to be transformed in the definitive IVS.[5] Furthermore, the same authors when analyzing the mitotic activity of myocytes during the development of the septum found two peaks of proliferation: the first aimed at the formation of trabecular outlines (stages 16-17HH) and the second after stage 26HH involved in the expansion of the ventricular chambers and elongation of the IVS.[6]

In regard to the molecular aspect, recent studies have shown that secreted signals of the endocardium towards the ventricular myocardium promote trabeculae formation.[7] One of the proteins directly involved is neuregulin 1 (NRG1), a protein of the epidermal growth factor family.

By *in vitro* culture studies it was found that NRG1 promotes the organization of sarcomeres and the concomitant differentiation of ventricular myocytes, both in adults and newborns.[8,9] It was also determined that NRG1 is synthesized by the atrial and ventricular endocardium and its action on the myocardium can be autocrine or paracrine processes. Transduction of NRG1 signal depends on ErbB receptors which, to be functionally active, should form homodimers (ErbB4/ErbB4) or heterodimers (ErbB2/ErbB4 or ErbB3/ErbB4).[10-12] Consistent with these findings, null mice of any of the NRG1 isoforms or their receptors (ErbB2, ErbB4) died before day 11 of embryogenesis due to poor differentiation of the ventricular myocytes and scarce trabecular outlines.[13] Also, by retrovirus inactivating the NRG1 function in chicken embryos, cardiac abnormalities similar to those of the null mice were induced.[14] In contrast, ErbB2 or ErbB4 deletion in the fetal ventricular myocytes, although permitting complete gestation newborn mice had a heart with dilated ventricles and poor contractile activity but without apparent involvement of the IVS.[15-17]

Despite all this information, the real role of NRG1 on the morphogenesis of trabecular outlines is yet to be defined. In order to investigate this aspect, studies of inhibition and induction of NRG1 activity in an organ-culture experimental model of chicken embryo hearts at stage 16-17HH were carried out, coinciding with the start of the development of trabecular primordia.

In order to inhibit the NRG1 activity an antibody and specific siRNA were used. Induction assays were carried out adding NRG1 α/β at different concentrations. Also, the effect of the NRG1 on the cyclic and apoptotic activity of the ventricular myocytes in the same period was determined.

It was discovered that mean concentrations of NRG1 have a positive effect on the initial trabeculogenesis, induces proliferation and prevents apoptosis of the ventricular myocardium. However, an increase in the NRG1 concentration does not favor early morphogenesis of the trabeculae. In contrast, it results in disorganized proliferation possibly by a molecular deregulation resulting from the excess of NRG1.

MATERIALS AND METHODS

Biological material

Fertile chicken eggs (*Gallus domesticus)* were incubated for 3 days at 38 °C and 86-87% humidity to obtain embryos at stage 16-17, according to the Hamburger and Hamilton classification that correlates the cardiac morphology with the age of the embryo.[18]

Antibodies

We used anti-neuregulin-1 IgG polyclonal goat antibodies (Santa Cruz Technology, Santa Cruz, CA), anti-PCNA (nuclear antigen of cellular proliferation), monoclonal rat IgG2a (BioGenex, Fremont, CA), and recombinant heregulin-β1 (NeoMarkers, Fremont, CA).

Embedding in paraplast

Samples for histological studies were fixed with alcoholic Bouin solution or neutral formalin for immunohistochem-

ical analysis. They were dehydrated with graded alcohols, made transparent with cedar oil, immersed in chloroform/paraffin (1:1) and embedded in paraplast. Frontal 5-um serial sections were cut using a microtome (Microm HM315, Thermo Scientific).

Histology

After deparaffinization and rehydration, slides were stained with hematoxylin and eosin (H/E). Microphotographs (10x and 100x) were taken using a digital capture system coupled to the microscope (Olympus BH2) with a 1.7x lens.

Organ culture

To establish the adequate culture conditions that would allow for a harmonious development of the trabecular region, stage 16HH chicken embryos were extracted from the shell and were placed in a physiological Ringer solution for birds (NaCl, KCl, $CaCl_2$). The hearts were dissected and washed with sterile PBS with penicillin-streptomycin 0.1%. Then they were incubated at 37 °C and 5% of CO_2 in 8-well culture plates in 0.3 ml liquid media (DMEM) supplemented with antibiotic and different concentrations (2%, 4%, 6%) of fetal bovine serum (FBS). They were incubated for 12 h. The culture media was then removed and the hearts were washed with 1X PBS and immediately fixed in alcoholic Bouin solution in order to make serial histological sections to evaluate trabecular development. All trials were carried out in triplicate. Chicken embryo hearts at stages 16HH to 18HH cultured *in ovo* were used to determine the histological characteristics of this organ and compare them to *in vitro* tissue cultures.

Induction with exogenous NRG1α/β

In order to determine the possible role of NRG1 in trabeculogenesis, we carried out an induction experiment of NRG1 activity. Based on the results of establishing culture conditions, hearts at 16-17HH stage were placed in 8-well culture plates with 0.3 ml of DMEM free of FBS, supplemented with antibiotics. First, endogenous NRG1 activity was completely inhibited, simultaneously adding anti-NRG1 (1:10) and siRNA-NRG1 (2 ng). The culture was incubated at 37 °C and 5% CO_2 for 2 h. The inhibited cultures were divided into batches to perform induction assays, adding recombinant heregulin-β1 at three different concentrations (2, 10, 20 ng/mL). One batch remained without the protein being added. The hearts were kept in culture for 12, 24 or 36 additional hours. Hearts that had been incubated in serum-free DMEM were used as controls. Once the material was harvested, it was fixed in alcoholic Bouin solution or neutral formalin and embedded in paraplast to analyze the histological features or determine the cyclic activity of the ventricular myocytes by immunodetection of the nuclear antigen of proliferation (PCNA).

Evaluation of the cyclic activity of ventricular myocytes

Based on the manufacturer's instructions (EnVision Stain System, Dako, Carpinteria, CA), slides with the samples were treated with a citrate solution under pressure (15 lb/in^2) to liberate the antigen. After lavage with PBS-Tween 20, endogenous peroxide activity was inhibited with hydrogen peroxide 0.1% (Dako). Following this, it was incubated from 5 to 10 min with a protein blocking solution (Biogenex) and with the primary antibody (anti-PCNA) for 30 min. After rinsing with PBS, the polymer coupled to horseradish peroxide (HRP) was incubated for 30 min. The complex was visualized with a solution of 3-3'diaminobenzidine (DAB) as chromogen. Slides were mounted with PBS/glycerol 1:1 and micrographs at 40x were taken using a digital capture system coupled to the microscope (Olympus BH2) with a 1.7x lens.

Analysis of the apoptosis

Hearts obtained from the NRG1 activity induction assays were incubated for 1 h at 37 °C in DMEM with Lysotracker (LTR) 1M (Molecular Probes, Eugene, OR). Then they were washed with 1X PBS, fixed with neutral formalin and embedded in paraplast to obtain 5-μm serial histological sections. The preparations were analyzed on a confocal microscope using a 594-nm excitation laser at 633 nm emission and the LSM 510 program.

RESULTS

Histological characteristics of the heart cultured *in ovo*

Chick embryo heart (stage 16-17HH) grown *in ovo* showed relatively thin ventricular myocardial wall formed by two- to three-cell thickness and covered internally by endocardium (Figures 1A-1B). Twelve hours later (stage

18HH) the ventricular wall showed two strata: a thin compact layer and small myocardial protrusions, surrounded by endocardium and projected to the ventricular lumen called trabecular outlines (Figures 1C-1D).

Organ culture conditions

Stage 16-17HH hearts cultured *in vitro* for 12 h showed a diverse tissue organization depending on the amount of FBS added to the media (Figure 2). Control hearts cultured in DMEM without FBS did not increase in size. However, although the ventricular myocardial wall thickness increased, it was less compact with small spaces free of myocytes on the surface opposite to the endocardium. Individual trabecular outlines did not become organized (Figure 2 AA'). FBS produced heart growth and increased the thickness of the ventricular myocardium. When the FBS concentration was increased, less myocardial compaction and greater disorganization was observed (Figures 2B-B', CC', DD').

Induction with exogenous NRG1α/β

Hearts at stage 16-17HH cultured in FBS-free DMEM were used as control to analyze the possible inductive action of NRG1 on the ventricular myocardium. When the hearts were cultured in that condition for 12 or 24 h, they were able to develop nascent trabecular outlines delimited by endocardium (Figures 3A-3B). However, by extending the incubation period to 36 h, the outlines showed loss of individuality and incipient histological disruption (Figure 3C). In contrast, by adding exogenous NRG1 (heregulin-β1), after total inhibition of NRG1 endogenous activity we found that the lowest concentration (5 ng) in cultures of 12 h was insufficient to induce increase in heart size or affect the thickness of the ventricular myocardial wall or trabeculogenesis (Figure 3D). At 24 h of incubation, the heart did not grow nor was there an increase in the thickness of the compact ventricular wall, but clearly evident fairly well-organized trabecular outlines were seen (Figure 3E). However, when the culture was prolonged up to 36 h, the hearts demonstrated a totally disorganized ventricular wall in which the compact myocardium or trabecular outlines were not distinguished (Figure 3F). The mean concentration of NRG1 (10 ng) in the 12-h cultures was sufficient to induce not only growth of the heart and marked increase in myocardial wall thickness, but also caused incipient trabeculogenesis with better organized trabecular outlines in 24-h cultures (Figures 3G-3H). In contrast, in 36-h cultures, although the size of the heart increased and ventricular wall was compacted, no traces of trabecular outlines were noted (Figure 3I). Finally, all cultures with 20 ng of NRG1 generated hearts with no trabeculogenesis. In this case, the multilayer compact ventricular myocardium was differentiated from the relatively lax and completely disorganized myocardium directly in contact with the ventricular lumen (Figures 3J-3K). In 36-h cultures, the compact wall of the ventricle was slightly less thick (Figure 3L).

Importance of the NRG1 on proliferation and apoptosis of the ventricular myocardium

With the goal of determining if NRG1 is involved in regulation of proliferation and apoptosis during formation of trabecular outlines and increase of the ventricular wall thickness, experiments of induction of the NGR1 activity were carried out. After 12 h, cyclic activity was evaluated using PCNA and apoptosis through labeling with LTR. Heart cultures with DMEM without FBS demonstrated histological characteristics similar to those previously

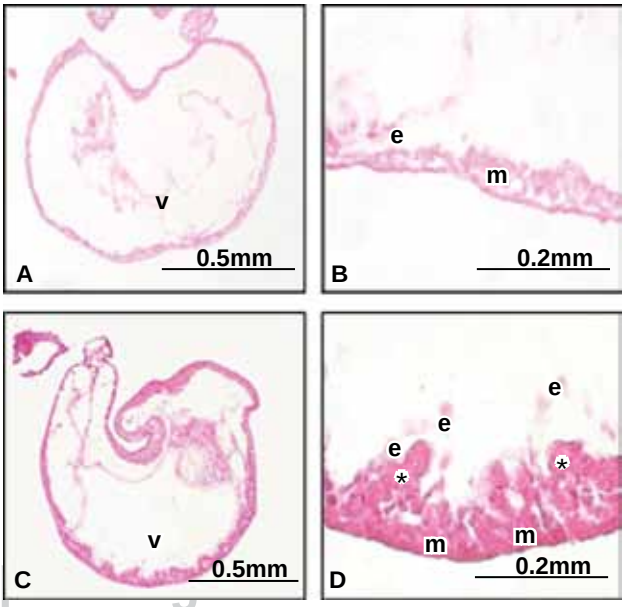

Figure 1. Morphological characteristics of the heart in stage 16HH (72 h of incubation) and 18HH (3-4 days of incubation). **(A,B)** Stage 16HH. Note in the ventricular region of the heart (v) the flat wall of the myocardium (m) with 2- to 3-cell thickness. **(C,D)** Stage 18HH. The ventricular region of the heart is comprised of compact myocardium (m) and small trabecular outlines. (*) Covered by endocardium (e).

described. They also manifested high positivity to PCNA and practically null labeling of LTR (Figures 4A-4D). However, in hearts in which endogenous NRG1 function was partially inhibited, in addition to presenting scarce trabeculogenesis, cyclic activity of the myocardium was severely reduced, and some foci of apoptosis began to be manifested (Figures 4E-4H). In contrast, when NRG1 activity was totally inhibited by simultaneously adding anti-NRG1 and siRNA, no indication of trabeculogenesis or cyclic activity of the myocardium were noted. However, LTR labeling showed a notable increase (Figures 4I-4L). Finally, when 10 ng of exogenous NRG1 was added to the totally inhibited cultures, trabeculogenesis was initiated accompanied by an elevated positivity to PCNA and practically null labeling of the LTR (Figures 4M-4P).

DISCUSSION

In chicken embryos, trabecular outlines begin to be formed shortly after the cardiac torsion and looping process is concluded, i.e., approximately during stage 16-17HH.[3,4,19] Initially, the trabeculated ventricular myocardium serves to increase oxygenation of the cardiac tissue before formation of the coronary arteries as well as to separate blood flow in the cardiac chambers before formation of the IVS. Also, the role that trabeculae play on the development of IVS has currently been documented.[5,6] In this manner, in extreme cases in which the trabeculae are not formed or they develop deficiently, early cardiogenesis is directly affected, causing premature death of the embryo. In contrast, abnormal development of the IVS gives rise to very complex ventricular septal defects. From there comes the importance of determining the molecular network that regulates trabeculogenesis.

Based on the above, the aim of the present study was to determine the role of NRG1 on trabeculogenesis and its possible function on regulation of proliferation and apoptosis of the ventricular myocardium, essential processes in the development of trabeculae and IVS.

Initially, an *in vitro* model of organ-culture of the chicken embryo heart (stage 16-17HH) was implemented. In this period was possible to analyze the inductive effect of NRG1 on trabeculogenesis because trabecular outlines are not yet developed. It was noted that heart cultured in DMEM without FBS manifested a delay of ~6 h with respect to what occurs *in ovo* and preserved the cytodynamic structure and characteristics similar to those of the heart in stage 17HH when the first indications of trabeculogenesis were observed (Fig 2A-A'). In contrast, FBS added to the organ-culture, in agreement with what has been reported in the literature, had a mitogenic effect on the ventricular cardiac myocytes.[20,21] Nevertheless, it caused a decompensated growth of the heart and caused loss of compactness and extreme disorganization of the myocardium of the ventricular wall. These results indicate a negative effect of FBS on trabeculogenesis, a fact that determined that in cultures of the NRG1 induction trials, FBS activity would not be used.

Once the organ-culture model was established, we examined the effect of exogenous NRG1α/β. Therefore, en-

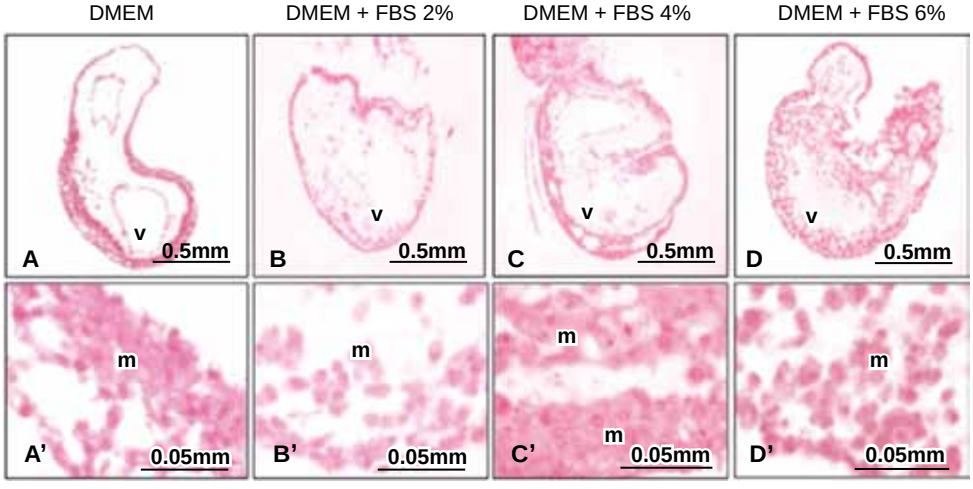

Figure 2. Effect of FBS added to the DMEM culture media in conditions of organ-culture for 12 h over the morphological characteristics of the ventricular trabeculated region (v) of the chicken heart stage 16HH. Micrographs acquired at 20x **(A-D)** and 40x **(A'-D')**. Note that the thickness of the wall and the disorganization of the myocytes (m) increases as the plasma concentration increases. The presence of endocardium is not distinguished.

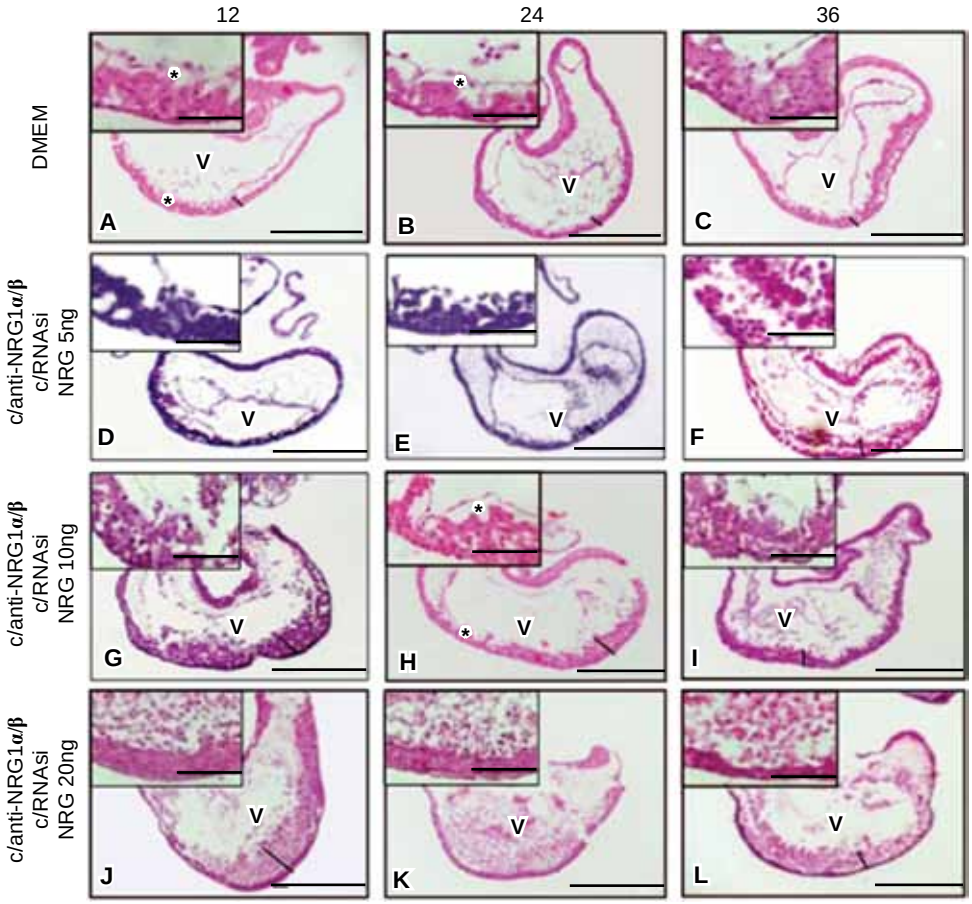

Figure 3. NRG1 effect on trabeculogenesis (*). Chicken embryo hearts in stage 16HH previously treated with anti-NRG1 and siRNA for totally inhibiting the activity of endogenous protein were incubated with different concentrations of exogenous NRG1 for 12, 24 or 36 h. Boxes show an amplification of the morphohistological characteristics of the ventricular region (v); the black line points to changes in wall thickness. **(A-C)** Control without treatment. **(D,E)** It is observed that 5 ng of NRG1 for short periods of time does not promote considerable changes. **(F)** Completely disorganized ventricular wall in which no compact myocardium or trabecular outlines are distinguished. **(G)** It is observed that at 10 ng for 12 h a growth in the size of the heart is induced with a notable increase of myocardial wall thickness. **(H)** 10 ng of NRG1 for 24 h is sufficient to promote trabeculogenesis (*). Incipient myocardium (m) outlines can be distinguished covered by endocardium (e). **(I-L)** Increase in protein concentration and longer incubation time favor proliferation of cardiac myocytes but not trabeculogenesis. Rod boxes = 0.2 mm; panoramic photos = 0.5 mm.

dogenous activity of the protein was inhibited first as well as the new protein synthesis, adding to the media a primary antibody and a specific siRNA. It was observed that the low concentrations of exogenous NRG1 (5 ng/ml) and the short culture time (12 h) were not sufficient to induce formation of trabecular outlines (Figures 3D-3F). Furthermore, from the beginning, high concentrations of exogenous NRG1 (20 ng/ml) generated the high growth of the heart but with obvious cellular disorganization. Moreover, trabeculae surrounded by endocardium were not formed. In its place, abundant highly disorganized myocytes were observed (Figures 3J-3L). In regard to the cultures with the addition of 10 ng/ml of NRG1, it was observed that the heart increased in size and preserved its morphology. The myocardial wall, in addition to manifesting high cyclic activity, showed trabecular outlines in development that maintained their individuality; however, this characteristic was lost when the heart was cultured for >24 h (Figures 3G-3I). These results reveal that, in agreement with the results of Gassmann et al. and Lee et al. in *knockout* mice,[12,13] intermediate NRG1 concentrations can have an inductive effect of initial trabeculogenesis, although it does not appear to be involved in the further development of the trabeculae because its effect was reduced in cultures that lasted >24 h. Another possibility is that the inductive capacity of the NRG1 on trabeculogenesis depends on the synergistic activity of this protein with the growth factor similar to insulin, also secreted by the endocardium as initially suggested by Herting et al.[22] and recently by Li et al.[23] The excessive addition of NRG1 did not provoke greater trabeculogenesis, although it did increase the mitotic activity of the myocardium. These findings allow us to suppose that excessive NRG1 causes a molecular disequilibrium that surpasses the morphogenic capacity of the myocardium to form trabeculae and favors a disorganized proliferation.

The analysis of the cyclic activity through immunodetection of the PCNA and the apoptosis by LTR on the organ-culture of hearts in stage 16-17HH revealed that the cyclic activity of the myocardium decreases when the NRG1 function is partially or totally inhibited, while cellular death increases (Figures 4G, 4H, 4K, 4L). In contrast, when overexpression of NRG1 was induced, an elevated mitotic activity and null apoptosis were noted (Figure 4O-4P). These results are in agreement with what has been observed by Zhao and Lemke in primary cultures of cardiomyocytes in neonates. These authors conclude that NRG1 induces proliferation of the myocardium and protects against apoptosis.[14]

The findings of this study show a significant role of NRG1 in the onset of trabeculogenesis promoting, besides proliferation of the myocardium, a protective activity against apoptosis. However, it is important to highlight that excessive NRG1 causes a molecular disequilibrium that could favor proliferation of the myocardium over the initial morphogenesis of ventricular trabeculae. Thus, understanding the role of NRG1 and its deregulation during early cardiogenesis, either by excess or shortage, continue to be a priority of basic research to provide data that reveal molecular networks involved in trabeculogenesis and further development of IVS.

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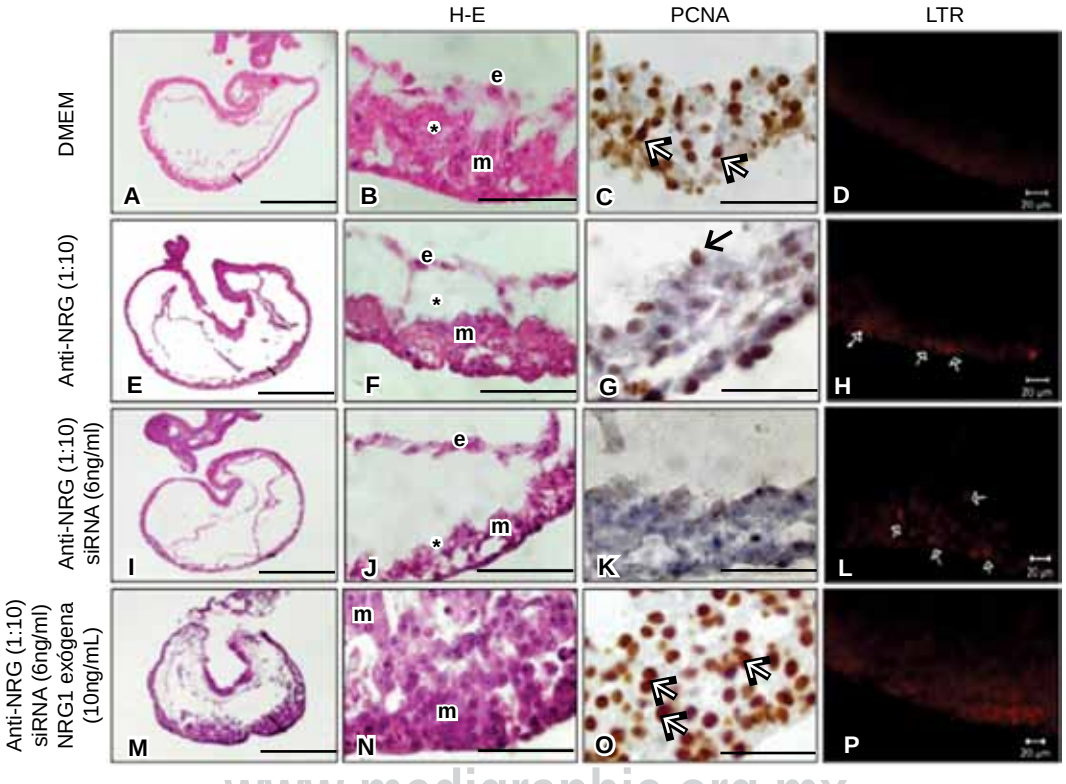

Figure 4. Implication of NRG1 in the proliferation and apoptosis of ventricular myocytes at the beginning of trabeculogenesis. **(A-D)** Organ-culture of heart in stage 16-17HH with DMEM for 12 h that manifests the beginning of trabeculogenesis **(A-B)** cyclical activity **(C)** and absence of apoptotic myocytes **(D)**. **(E-H)** Partial inhibition of NRG1 activity with poor trabeculogenesis **(E,F)**, decrease in cyclic activity of myocytes **(G)** and few apoptotic foci **(H)**. **(I-L)** Total inhibition of the NRG1 activity causes almost null trabeculogenesis **(I-J)**, scarce cyclical activity of the ventricular myocytes **(K)** with slight increase of apoptotic foci **(L)**. **(M-P)** Addition of the NRG1 favors growth of ventricular chamber walls (v) without forming incipient trabecular outlines **(M,N)**, through increase of cyclical activity of the myocytes **(O)** and reduction in cellular death **(P)**. Black arrows show positive nuclei to proliferation antigen (PCNA). White arrows point to apoptotic foci detected by LTR. e, endocardium; m, myocardium. *Zones of trabeculogenesis. Panoramic rod photos = 0.5 mm; amplifications = 0.2 mm.

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