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Cardiospecific knockout of αE-catenin leads to violation of the neonatal cardiomyocytes maturation via β-catenin and Yap signaling

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Aim. To study the αE-catenin gene function in neonatal cardiomyocytes proliferation and maturation. **Methods.** Conditional knockout approach, histological (H&E and acridine orange staining) and molecular genetics (qPCR) methods were used. Result. The hetero- and homozygous embryonic cardiospecific knockout of αE-catenin is associated with an increased level of neonatal cardiomyocytes proliferation and a decreased binucleated cells frequency. Knockout of αE-catenin leads to up-regulation of the β-catenin- and Yap-target genes expression (*Axin2, c-Myc, Tcf-4, CyclinD1, Ctgf and Tnfrsf1b*). **Conclusion.** αE-catenin is involved in the regulation of proliferation and maturation of neonatal cardiomyocytes via modulation of the activity of β-catenin- and Yap-dependent transcription.

K e y w o r d s: heart maturation, αE-catenin, Wnt signaling, HIPPO signaling, cardiomyocyte

Introduction

Cardiogenesis, heart maturation and function are complex processes regulated by multiple signaling pathways and maintained by cell adhesion molecules. During last decades it was shown that many classical adhesion proteins are also involved in the regulation of signaling cascades and have an important function in the mechanical cell coupling and determination of cell fate [1, 2]. αE-catenin, one of such proteins, is a classical component of the cell-cell adhesion that links the cadherin-catenin complex to the actin cytoskeleton [1, 3]. A structural function of αE-catenin has been studied using experimental murine models and isolated cells. It was shown that αE-catenin is critically required for early embryonic development at the blastocyst stage [4]. Interesting, that heart-specific knockout of αE-catenin did not affect cardiogenesis or embryonic development [5]. In adult heart the cardiospecific knockout of αE-catenin was found to lead to cardiomyopathy and intercalated disc abnormalities [6].

Recently the signaling function of αE-catenin has been described. Knock out of αE-catenin in keratinocytes leads to their hyperproliferation due to releasing of Yap from cyto-

solic trap and translocation to the nucleus [7]. α-Catenin suppresses the transcriptional activity of Yap in adult heart too. Thus, double knockout of αE- and αT-catenin in heart induces the Yap-dependent transcription and cardiomyocytes proliferation [8]. Moreover, α-catenin is able to modulate canonical Wnt signaling in cells. It prevents the interaction between the β-catenin/T-cell factor complex and DNA, and stimulates the β-catenin degradation [9,10]. Therefore, all these experimental data suggest a dual function of αE-catenin, structural and signaling, and indicate its more complex role in the heart remodeling and function. Our recent experimental data also support this idea. We have found that cardiospecific knockout of αE-catenin caused enlargement of the heart, fibrosis, upregulation of hypertrophic genes and led] to adult mice lethality after [the age of 10 months [11]. Moreover, we have established that the αE-catenin ablation induces the β-catenin- and Yap-dependent transcription in cardiomyocytes [12]. It is widely known that Wnt- and HIPPO-signaling is important for cardiomyocytes survival and proliferation, heart size and metabolism regulation, hypertrophic response and heart failure development [13–16]. Altogether, this indicates that the αE-catenin gene dysfunction in embryonic heart leads to the pathological changes in heart, heart dysfunction and lethality through the canonical Wnt- and HIPPO-signaling modulation. Nevertheless, the time of the beginning of pathological changes in the αE-catenin deficient hearts is unknown. Therefore, in the present study we have focused on the analysis of activity of the canonical Wnt- and Hippo-signaling pathways in neonatal cardiomyocytes. For this, we have used the conditional knockout approach and analyzed the newborn heart and cardiomyocytes morphology as well as the canonical Wnt- and Yap-target gene expression in the isolated neonatal cardiomyocytes with hetero- and homozygous knockout of αE-catenin.

Materials and Methods

Mice generation. To obtain mice with heterozygous and homozygous cardiac-specific αE-catenin deletion we mated αE-cat$^{flox/flox}$; αMHC-Cre^{-} mice with αE-cat$^{flox/wt}$; αMHC-Cre^{+}. Offsprings of following genotypes were used: αE-cat$^{flox/wt}$; αMHC-Cre^{+} (heterozygous), αE-cat$^{flox/flox}$;α-MHC-Cre^{+} (homozygous), and αE-cat$^{flox/wt}$;αMHC-Cre^{-}, αE-cat$^{flox/flox}$; α-MHC-Cre^{-} (controls). All animals were used in accordance with the Council Directive on the approximation of laws, regulations, and administrative provisions of Member States regarding the protection of animals that are used for experimental and other scientific purposes (86/609/EEC). All experimental procedures were approved by the Institute of Molecular Biology and Genetics of National Academy of Science of Ukraine Ethical Committee.

Mouse genotyping. Tail tip tissues were used for genotyping. DNA isolation and polymerase chain reaction (PCR) were performed. Sequences of primers for genotyping were published earlier [5].

Morphometric analysis of new born heart. After the one-day old animals were sacrificed, body weight (BW, g) and heart weight (HW, mg) were measured. These parameters were used to calculate the HW/BW ratio.

Neonatal cardiomyocytes isolation and culture. The mouse pups aged 1 day were decapitated, whole heart were excised and

weighted. The atria were dissected and ventricles were transferred to DMEM with 10% FBS and 10x penicillin and streptomycin (1000U/ml and 1000μg/ml respectively) for 30 min. Then DMEM was replaced with 0,025% Trypsin and 0,01% EDTA in PBS and left overnight at +4° C. Next day, after trypsinization, hearts were disintegrated by pipetting and a cell suspension from each heart was plated in DMEM with 10% FBS into wells of 12-well plate. The cells were incubated in a humid 5% CO2 incubator at 37° C.

H&E and acridine orange staining. Medium was discarded; cells were washed twice with PBS, fixed in 4% paraformaldehyde for 10 min and stained with hematoxylin and eosin (HE). The cells were analyzed using light microscopy Primo Star (Carl Zeiss, Göttingen, Germany) and AxioVision Software (Carl Zeiss, Göttingen, Germany). Length (the longest axis of the cell) and width (perpendicular to the length at the level of nuclei) of cardiomyocytes were determined. For nuclear detection cardiomyocytes were washed twice with PBS, then incubated for 10 min with 20 μg/ml acridine orange solution in PBS, washed three times with PBS and fixed in 4% paraformaldehyde for 10 min. Cells were analyzed using Leica DM 1000 fluorescent microscope (Leica Microsystems, Wetzlar, Germany).

MTT-assay. Approximately 10000 of cardiomyocytes were seeded into 96-well plate and cultured for 48 h at 37° C, 5% CO2. Then cells were incubated with 15 μl MTT solution (5 mg/mL, Sigma, USA) for 4 h at 37° C, 5% CO2. The formazan precipitates were dissolved in 100 μl DMSO and the absorbance at 570 nm of each well was measured by spectrofluorometer Synergy HT (BioTek, USA).

Total RNA isolation, cDNA synthesis and qPCR. Cells were lysed with innuSOLV RNA Reagent (Analytik Jena AG, Germany) according to the manufacturer's instructions. RNA concentration and purity were determined with (NanoDrop Technologies Inc., USA). 1 μg of total RNA was treated with DNAse I (Thermo Fisher Scientific, USA) according to the manufacturer's recommendations. cDNA was synthesized with Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. PCR was performed with Maxima SYBR Green/Fluorescein qPCR Master Mix (Thermo Fisher Scientific, USA) using the CFX96 (BIO-RAD, USA). The gene expression is represented as the ΔC_T value normalized to the reference gene *GAPDH*. The $\Delta\Delta C_T$ value for each target gene was calculated by subtracting the average ΔC_T from the control group. Finally, the *n*-fold difference was calculated using $2^{-\Delta\Delta CT}$ method. Sequences of primers for qPCR are available elsewhere [12].

Statistical analysis. Data on the graphs represent mean ± standard deviation. Differences between groups were evaluated using one-way analysis of variance (ANOVA) followed by Holm-Sidak multiple-comparison *post hoc* test. Values of $p < 0.05$ were considered statistically significant. The statistical analyses were performed using Prism7 software (GraphPad, USA).

Results and Discussion

Recently we have shown that embryonic cardio-specific knockout of αE-catenin leads to the heart enlargement, tissue architecture remodeling, up-regulation of hypertrophic genes with age and adult mice lethality [11]. Here we have focused on the influence of hetero- and homozygous

knockout of αE-catenin on the heart of newborn mice and isolated neonatal cardiomyocytes.

We have found that homozygous knockout of αE-catenin in heart after 7.5 days of embryonic development leads to a significantly higher HW/BW ratio in the mutant new born mice (Fig. 1A). This finding is in agreement with our and others data [8, 11] where a significantly higher HW/BW ratio in the adult heterozygous and homozygous αE-catenin mice was shown. This data suggest that cardiospecific ablation of αE-catenin in embryonic hearts leads to a significant enlargement of newborn and later of adult heart.

Next, we have analyzed how αE-catenin knockout affected the cardiomyocytes size and their proliferation. For this we have utilized the isolated neonatal cardiomyocytes which were cultured under the standard conditions. MTT-assay indicated a significantly higher

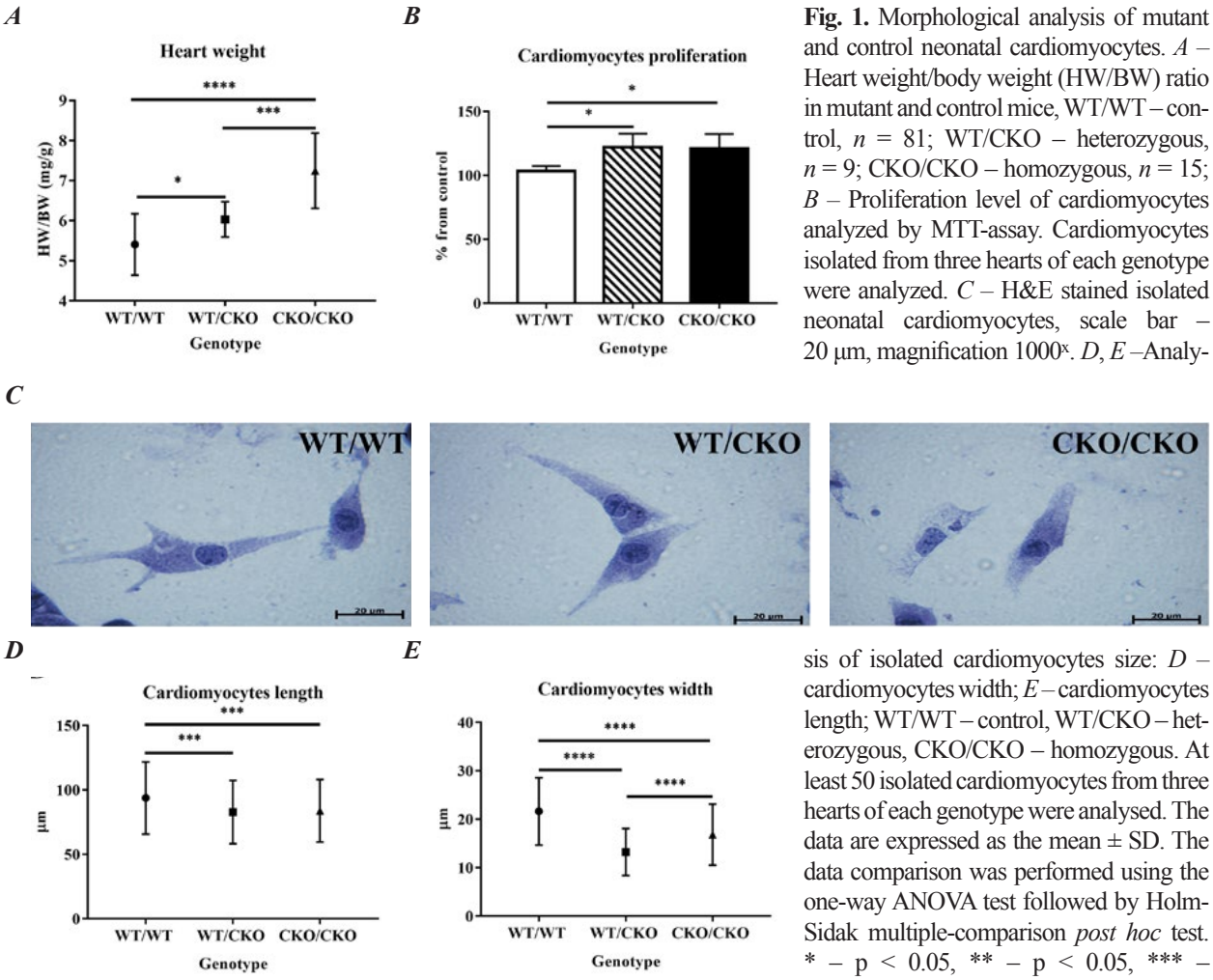

Fig. 1. Morphological analysis of mutant and control neonatal cardiomyocytes. *A* – Heart weight/body weight (HW/BW) ratio in mutant and control mice, WT/WT – control, $n = 81$; WT/CKO – heterozygous, $n = 9$; CKO/CKO – homozygous, $n = 15$; *B* – Proliferation level of cardiomyocytes analyzed by MTT-assay. Cardiomyocytes isolated from three hearts of each genotype were analyzed. *C* – H&E stained isolated neonatal cardiomyocytes, scale bar – 20 µm, magnification 1000^{x}. *D*, *E* –Analysis of isolated cardiomyocytes size: *D* – cardiomyocytes width; *E* – cardiomyocytes length; WT/WT – control, WT/CKO – heterozygous, CKO/CKO – homozygous. At least 50 isolated cardiomyocytes from three hearts of each genotype were analysed. The data are expressed as the mean ± SD. The data comparison was performed using the one-way ANOVA test followed by Holm-Sidak multiple-comparison *post hoc* test. * – $p < 0.05$, ** – $p < 0.05$, *** – $p < 0.005$, **** – $p < 0.001$.

level of the mutant cardiomyocytes proliferation (Fig. 1B). The analysis of length and width of isolated cardiomyocytes revealed that hetero- and homozygous deletion of αE-catenin significantly reduced the width of mutant cells compared to the control cells (Fig. 1C). The length of mutant cells also was less compared to the control cardiomyocytes (Fig. 1D). This data suggest that cardiomyocytes with hetero- and homozygous knockout of αE-catenin proliferate more actively and this is associated with a smaller size of cells. Thus, the αE-catenin ablation in embryonic heart probably leads to the cell cycle disturbance.

Widely known that during cardiogenesis in all species, including rodents, all cardiomyocytes are mononuclear and diploid [17, 18]. But in adult mice cardiomyocytes mostly become binucleated and polyploid after birth, this happens between P1-P7 [17] and is a sign of heart maturation Taking into account the changes in cells size and a higher proliferation rate of mutant cardiomyocytes, we considered this as a violation of heart maturation under the αE-catenin knockout conditions. Therefore, next we have analyzed the frequency of binucleated isolated cells in both groups of mutant cardiomyocytes compared to the control cells (Fig. 2A). Using acridine orange staining, we have revealed that the hetero- and homozygous knockout of αE-catenin significantly decreased the frequency of binucleated cardiomyocytes compared to the control (Fig. 2B). These data indicated that the αE-catenin ablation negatively affects cardiomyocytes maturation probably via its signaling function, because earlier we have reported that in an embryonic heart αE-catenin knockout is compensated by αT-catenin in AJ [5].

An analysis of the hypertrophic or fetal genes expression revealed a significantly higher level of the *ANP* gene expression in both groups of mutant neonatal cells (Fig. 3A). The number of other hypertrophic genes, *BNP* and *β-MHC*, was

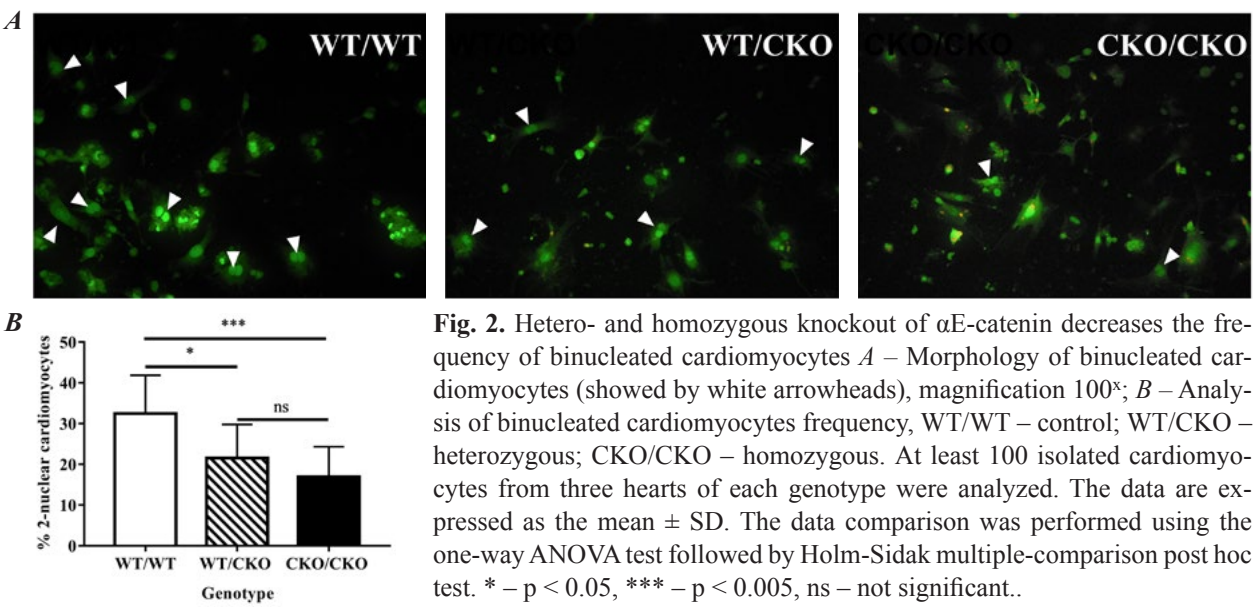

Fig. 2. Hetero- and homozygous knockout of αE-catenin decreases the frequency of binucleated cardiomyocytes *A* – Morphology of binucleated cardiomyocytes (showed by white arrowheads), magnification 100^x; *B* – Analysis of binucleated cardiomyocytes frequency, WT/WT – control; WT/CKO – heterozygous; CKO/CKO – homozygous. At least 100 isolated cardiomyocytes from three hearts of each genotype were analyzed. The data are expressed as the mean ± SD. The data comparison was performed using the one-way ANOVA test followed by Holm-Sidak multiple-comparison post hoc test. * – $p < 0.05$, *** – $p < 0.005$, ns – not significant..

significantly elevated only in the αE-catenin haploinsufficient cardiomyocytes (Fig. 3A). Interestingly, in the adult αE-catenin mutant hearts we have registered a significant elevation of the *ANP* and *β-MHC* genes (unpublished data). Probably, the αE-catenin ablation negatively affects the postnatal heart development and leads to the fetal up-regulation at aging.

Our own and foreign published data suggest that αE-catenin is involved in the cell cycle control in new born heart via its signaling function [8, 12]. Probably mutant cardiomyocytes proliferate actively and this leads to a decrease in both cell size and binucleated cells frequency. Earlier we have shown that αE-catenin ablation leads to the activation of β-catenin and Yap dependent transcription in adult heart [12]. Regulation of cardiomyocytes and keratinocytes proliferation by αE-catenin via Yap and MAPK pathways was demonstrated experimentally [7, 8, 19]. Noteworthy, HIPPO, Wnt/β-catenin and MAPK pathways, among others, determine the cell fate, and participate in the progression of heart dysfunction and the development of heart failure [20, 21]. Therefore, we suggested that the mice lethality, morphological abnormality and molecular changes in adult mice with the cardiospecific ablation of αE-catenin [11, 12] may result from the disturbance of the αE-catenin signaling function.

Thus, next we have analyzed the signaling activity of β-catenin and Yap in neonatal cardiomyocytes. We have found a significant elevation of the β-catenin and Yap transcriptional activity in the αE-catenin hetero- and homozygous neonatal cardiomyocytes (Fig 3B and 3C). In neonatal cardiomyocytes we observed a significantly higher level of expression only of the two Yap target genes (*Ctgf* and *Tnfrsf1b)* (Fig. 3C) whereas in adult mice all studied Yap target genes were overexpressed [12]. Interestingly that in the present study we have observed a higher level of some β-catenin and Yap target genes expression in the αE-catenin heterozygous new born mice compared with the αE-catenin CKO/CKO hearts (Fig. 3B and 3C). Some fetal genes

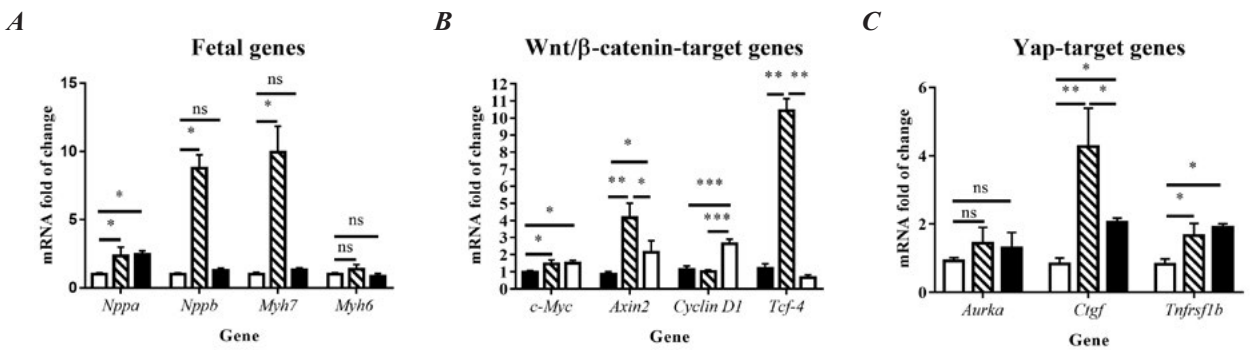

Fig. 3. *A* – Expression of the hypertrophic genes in isolated neonatal cardiomyocytes. *B* – Expression of the Wnt/β-catenin target genes in isolated neonatal cardiomyocytes; *C* – Expression of the Yap target genes in isolated neonatal cardiomyocytes. The data comparison was performed using one-way ANOVA followed by Holm-Sidak multiple-comparison *post hoc* test. The data are expressed as the mean ± SD of arbitrary fold increases relative to control levels. $n = 3$/group. *$p < 0.05$, **$p < 0.01$, *** < 0.005, ns – not significant.

(*BNP* and *β-MHC*) also were elevated in neonatal cardiomyocytes with the heterozygous knockout of αE-catenin, but not in cardiomyocytes with the homozygous knockout of αE-catenin (Fig. 3A). Therefore, the cardiospecific αE-catenin knockout leads to the β-catenin and Yap signaling activation in neonatal cardiomyocytes.

Conclusions

The embryonic cardiospecific ablation of αE-catenin stimulates the proliferation of neonatal cardiomyocytes and attenuates their maturation (smaller cardiomyocytes and a lower level of binucleated cardiomyocytes) via the β-catenin- and Yap-dependent transcription activity. The signaling function of αE-catenin is crucially important for the postnatal heart maturation and function. Our and foreign published data indicate that the disturbance in the αE-catenin signaling function in neonatal heart resulted in the adult heart pathology and mice lethality.

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Кардіоспецифічний нокаут αE-катеніна призводить до порушення формування неонатальних кардіоміоцитів через β-катеніновий та Yap сигналінги

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Мета. Дослідити функуцію гена αE-катеніна в проліферації таи формированні неонатальних кардіоміоцитів. **Методи.** В роботі використовували метод умовного нокаута гена, гістологічні і молекулярно-генетичні методи. **Результати.** Гетеро- і гомозиготний кардіоспецифічний нокаут αE-катеніна асоційований із підвищенням рівня проліферації неонатальних кардіоміоцитів, збільшенням розмірів серця та зниженням частоти двоядерних кардіоміоцитів. Нокаут αE-катеніна призводить до зростання рівня експресії генів мішеней β-катеніна и Yap (*Axin2*, *c-Myc*, *Tcf-4*, *CyclinD1*, *Ctgf* и *Tnfrsf1b*). **Висновки.** αE-катенін залучається до контролю проліферації та формування неонатальних кардіоміоцитів через регуляцію транскрипційної активності β-катеніна та Yap.

Ключові слова: формування міокарда, αE-катенін, Wnt сигналінг, HIPPO сигналінг, кардіоміоцити

Кардиоспецифический нокаут αE-катенина вызывает нарушение формирование неонатальных кардиомиоцитов посредством β-катенинового и Yap сигналингов

В. В. Балацкий, Т. П. Рубан, Л. Л. Мацевич, О. А. Пивень

Цель. Изучить функцию гена αE-катенина в пролиферации и формировании неонатальных кардиомиоцытов. **Методы.** В работе использовали метод условного нокаута гена, гистологические и молекулярно-генетические методы. **Результаты.** Гетеро- и гомозиготный кардиоспецифический нокаут αE-катенина ассоциирован с повышением уровня пролиферации неонатальных кардиомиоцитов, увеличением размера сердца и снижением частоты двуядерных кардиомиоцитов. Нокаут αE-катенина вызывает экспрессию генов мишеней β-катенина и Yap (*Axin2*, *c-Myc*, *Tcf-4*, *CyclinD1*, *Ctgf* и *Tnfrsf1b*). **Выводы.** αE-катенин вовлечен в контроль пролиферации и формирования неонатальных кардиомиоцитов посредством регуляции β-катенивой и Yap-зависимой транскрипционной активности.

Ключевые слова: формирование миокарда, αE-катенин, Wnt сигналинг, HIPPO сигналинг, кардиомиоциты

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