Original Research

miR-1, *miR-133a/b,* and *miR-208a* in human fetal hearts correlate to the apoptotic and proliferation markers

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Abstract

The heart is the first organ to function in the developing embryo. MicroRNAs (miRNAs) are small non-coding RNAs involved in the translational regulation of gene expression, which is beside transcriptional regulation crucial for the morphologic development of muscle tissue. The aim of our study was to test the hypothesis that the expression of *miR-1*, *miR-133a/b,* and *miR-208a* correlates with gestational age as well as with an apoptotic and proliferative index in the developing human heart. Our study included normal heart tissue samples obtained at autopsy from 46 fetuses, 12 children, and 15 adults. Proliferation and apoptosis were measured by the immunohistochemical detection of Ki67 and cleaved-CK18. Expression of *miR-1*, *miR-133a*, *miR-133b*, and *miR-208a* was measured using real-time PCR. We found a similar level of expression of *miR-133a/b* in fetal and children hearts that was different from the levels in healthy adults. We also found a correlation between a *miR-208a* expression to the gestational age of fetuses. We observed an inverse correlation between Ki67 expression and gestational age. Expression of Ki67 was positively correlated to the expression of *miR-208a* and *miR-1*, but inversely correlated to the expression of *miR-133a/b.* Expression of cleaved-CK18 was also inversely correlated to the expression of *miR-133a/b*. Our results showed a general decrease in the expression of *miR-1* and an increase of *miR-133a/b* with increasing gestational age. We also found a general decrease in the expression of *miR-208a,* mimicking the expression of its host gene. Our results also suggest the involvement of *miR-208a* and *miR-1* in the proliferation as well as anti-proliferative and anti-apoptotic roles of *miR-133a/b*.

Keywords: Human developing heart, miRNAs, M30, Ki67, expression patterns

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Introduction

The heart is the first organ to function in the developing embryo. Cardiac development depends on an intricate coordination of different cell types; therefore strict regulation is essential at critical temporal points. Cardiomyocytes represent the majority of the cell types constituting the heart and are terminally differentiated. Their development is a coordinated process of cellular proliferation, migration, differentiation, and cell death (apoptosis). Several key elements orchestrate development of cardiomyocytes and other heart tissues, and microRNAs (miRNAs) seem to be one of them. miRNAs are small non-coding RNAs involved in the translational regulation of gene expression, which is beside transcriptional regulation crucial for morphologic development of muscle tissue.[1] miRNAs are therefore believed to be involved in the developmental, physiological, and pathological aspects of cardiology.

One of the first studies to suggest the importance of miRNAs in cardiogenesis was the pre-natal cardiac-specific deletion of Dicer, an enzyme responsible for processing of miRNA precursors into mature and functional miRNAs.[2] Loss of Dicer resulted in a dramatic decrease in the amount of mature miRNAs, defects in heart development and embryonic lethality.[2,3] Deletion of Dicer at a later developmental stage resulted in post-natal lethality, further supporting the idea that Dicer and miRNAs are essential for cardiac development and function. All mice lacking Dicer died within four days after birth; and their hearts were substantially larger than that of the control group whereas histological analyses indicated dramatic left ventricle dilatation.[3]

The majority of miRNAs are ubiquitously expressed. However, a number of miRNAs have been found to be expressed in a tissue-specific manner including muscle-specific and cardiac-specific expression patterns.[1,4] Tissue-specific expression of miRNAs is likely regulated at the transcriptional level, suggesting that tight temporal and spatial regulation of miRNA expression is important for

their function.[5] Serum response factor (SRF) is a cardiac-enriched transcriptional factor (TF) responsible for the regulation of organized sarcomeres in the heart.[6,7] SRF binds to CArG motifs in the promoters and enhancers of the muscle-specific genes that regulate differentiation, cell-cycle progression and tissue specific-gene expression.[7–9] Several miRNAs are known to be regulated by SRF, which is a their weak activator, but cooperatively induces respective miRNAs together with other TFs and cofactors.[6] In the heart, SRF binds and activates the enhancer region of *miR-1* and *miR-133a*.[8,9] *miR-1* and *miR-133a* are expressed as a bicistronic cluster in cardiac and skeletal muscle, and are transcriptionally regulated by the myogenic differentiation factors SRF/myocardin and MyoD/Mef2.[2,5,10] On the other hand, *miR-133b* is believed to only be expressed in skeletal muscles. However, we and others have shown that it is also expressed in human heart and cardiomyocyte cell lines.[11,12] Both *miR-1* and *miR-133* are believed to be expressed early in embryonic development, having similar functions regulating proliferative and apoptotic activities, while having opposite roles later in embryonic development. *miR-208a* is expressed together with its ghost gene, *Myh6,* that encodes alpha-myosin heavy chain (alpha-MHC).[13] There is evidence that the expression of cardiac-specific *miR-208a* is also developmentally regulated in the heart. It has been suggested that *miR-208a* is required to maintain the expression of cardiac TFs that are important for the development of conduction system.[14] miRNA *miR-1*, *miR-133a/b,* and *miR-208a* are believed to be involved in cardiomyocyte proliferation and differentiation; and other functions have been also attributed to the *miR-1* and *miR-133a/b,* such as regulation of cardiomyocyte apoptosis.[1,15,16] However, heart development not only depends on the absolute concentration of certain miRNAs, but also on their correct spatiotemporal expression.[6]

In our previous study, it has been shown that apoptosis in the developing heart resembles apoptosis in epithelial tissues and that the apoptotic index progressively decreases with increasing gestational age.[17] In the present study, we analyzed the hypothesis that the expression of *miR-1*, *miR-133a/b,* and *miR-208a* is in correlation with gestational age, as well as with apoptotic and proliferative indexes in the developing human heart, as analyzed by immunohistochemical expression of cleaved-CK18 and Ki67, respectively. To the best of our knowledge, this is also the first study to describe the expression of these miRNAs in human fetal hearts of different gestational ages.

Materials and methods

Patients and tissue samples

The study included 45 fetal heart samples from autopsy at gestational ages of 13–41 weeks that died *in utero*. The number of fetuses and their corresponding gestational age are summarized in Table 1. Fetuses were aborted either spontaneously (17) or by parents' choice (8). In cases of intrauterine fetal death (21), no evident cause of death was identified at autopsy. The terminations were performed according to Slovenian law. There were no signs of intrauterine growth retardation, and no macroscopic or microscopic evidence of disease or congenital anomalies at autopsy. Cases, in which macroscopic or microscopic evidence of disease or congenital anomaly was found at autopsy, were not included in this study. Autopsies were performed within 24 h of death.

Table 1 Number of fetuses of different gestational age included in study

Gestational week	Number of fetuses	Gestational week	Number of fetuses
13	4	29	1
14	1	32	2
16	2	33	1
17	1	34	1
18	6	35	2
19	3	36	2
20	1	37	2
21	1	38	2
22	1	39	5
23	2	40	2
24	1	41	2

The control group consisted of heart tissue obtained at autopsy from 13 children (4 children aged up to 3 months, 3 children 4–12 months old, and 6 children 1–5 years old). There were no preterm infants, infants with a maternal history of diabetes mellitus, or patients with a history of chemotherapy, viral infection, congenital heart disease, or chromosomal anomalies. The postmortem delay did not exceed 24 h, and there was no macroscopic or microscopic evidence of disease at autopsy.

The control group also included heart tissue obtained at autopsy from 15 healthy adults who died in accidents (aged 18–30, mean age 23.3 ± 4.8 years). There were no microscopic or macroscopic evidence of disease at autopsy. Death occurred within 30 min. Autopsies were performed within 24 h of death.

All tissue samples were fixed in 10% buffered formalin, embedded in paraffin (FFPE) and cut at 4 μm for H&E slides. The study was approved by the National Medical Ethics Committee and was carried out according to the Declaration of Helsinki.

Immunohistochemistry

FFPE tissue samples were cut at 4 μm for immunohistochemistry. We used commercially available antibodies against cleaved-CK18 (M30, 10700, Peviva, Sundbyberg, Sweden), and Ki67 (MIB-1, M7240, Dako by Agilent, Santa Clara, CA). Deparaffinization, antigen retrieval, and staining with antibodies were performed in an automatic immunostainer (Discovery or Benchmark XT, Ventana by Roche, Basel, Switzerland) in combination to treating sections with secondary antibody and color development with horseradish peroxidase (iVIEW DAB Detection Kit, Ventana, Basel, Switzerland). The sections were then counterstained with hematoxylin. Tissue sections of colonic adenocarcinoma served as a positive control and sections treated without

primary antibodies served as negative controls. The densities of cleaved-CK18 positive cells and Ki67-positive cells were estimated using an image analysis system (Cell and Tissue Analysis, Leica, Wetzlar, Germany) and expressed as the number of positive cells per mm^2. Three samples were analyzed from each case. After measuring the surface area of a high power field (HPF), we counted the total number of positive cells in 20 HPFs, or fewer in smaller hearts. The density was calculated by dividing the number of positive cells in 20 HPF with the surface area of a HPF $\times$ 20.

RNA isolation

Tissue samples were cut at 10 μm from FFPE tissue blocks. Three to eight 10 -μm sections were used for the isolation procedure. Total RNA isolation was performed using a miRNeasy FFPE kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol. All of the reagents were from Qiagen, except where otherwise indicated. Briefly, after deparaffinization using Xylene (Merck, Darmstadt, Germany) and an ethanol-washing step (Merck, Darmstadt, Germany), the pellets were air-dried and digestion with Proteinase K was performed at 55°C for 15 min, followed by 15 min of incubation at 80°C in order to partially reverse the formaldehyde modification of the nucleic acids. After the gDNA elimination step and the addition of ethanol to the samples, the mixture was transferred to an RNeasy MiniElute spin column. After two washing steps, the RNA was eluted in 30 μL of nuclease-free water. The concentration of the extracted RNA was measured using a NanoDrop-1000 (Thermo Fisher Scientific, Waltham, MA) and tested for UV/Vis ratio. The A_{260}/A_{230} intensity ratio needs to be above 1.0 and A_{260}/A_{280} needs to be above 1.8. The integrity and presence of the small RNAs (<200 nucleotides) was analyzed on a Bioanalyzer 2100 (Agilent) using SmallRNA Chips (Agilent).

miRNA reverse transcription and TaqMan-based qPCR analysis

Looped primers for specific miRNA reverse transcription (RT) including *hsa-miR-1*, *hsa-miR-133a*, *hsa-miR-133b*, and *hsa-miR-208a* were utilized following the manufacturer's protocol. *hsa-miR-26b* served as the internal control gene as previously suggested, described, and validated.[11,18,19] All of the reagents and instruments were from Applied Biosystems (Life Technologies, Carlsbad, CA), except where otherwise indicated. Briefly, the 15 μL RT reaction master mix was performed with 10 ng of the total RNA sample. qPCR was carried out using the Applied Biosystems 7900 Real-Time PCR System in a 20 μL PCR master mix containing 10 μL TaqMan 2 x Universal PCR Master Mix, 1 μL TaqMan assay, and 9 μL RT products diluted 10-fold. The qPCR reactions were performed in triplicate and the signal was collected at the endpoint of every cycle. In addition to qPCR analysis, a pool of RNA samples was created which was obtained from FFPE tissue samples. After RT, the cDNA was diluted in five steps, ranging from 3-fold dilution to 243-fold dilution, and the probes were tested for PCR efficiency. All of the qPCR efficiency reactions were performed in triplicate.

Statistical analysis

Based on the results of the efficiency analysis of the probes, the relative expression was presented as the $2^{-\Delta\Delta Cq}$ method.[20] The gene of interest (Cq_{GOI}), *miR-1, miR-133a/b, miR-208a* was calculated relative to the reference gene (Cq_{RG}), *miR-26b* (ΔCq). Because of the small groups of samples for all statistical analyses non-parametric tests were used. The calculated ΔCq were compared and tested for statistical significance between multiple groups of samples (i.e. fetuses, children, healthy adults) using the Kruskal–Wallis and between two groups of samples using Mann–Whitney tests (i.e. fetuses in comparison to healthy adults; differences between gender). The data were presented as a fold change in a graph with error bars representing the calculated fold change error using the SD of the Cq triplicates. A Spearman correlation coefficient was used to evaluate the correlation between gestational age and the density of Ki67, as well as was to test the correlation between the expression of *miR-1, miR-133a/b, miR-208a* and the density of cleaved-CK18, Ki67, and gestational age, and between expression of miRNAs themselves. Data were represented as Spearman rank correlation coefficient with corresponding P value as well as in graphs. SPSS analytical software ver.20 (IBM Corp., Armonk, NY) was used for all statistical analyses with a cut-off at $P < 0.05$.

Results

Patients and tissue samples

We have performed statistical analysis between groups of samples with different causes of fetal abortion. There was no difference in miRNAs expression between fetal heart tissues that was obtained after spontaneous abortion or after abortion by parents' choice. Both groups were of similar gestational age (13–18 and 13–22, respectively). There was no statistical significant change in expression of muscle-specific miRNAs between heart tissue of female and male fetuses and between heart tissue from healthy adult men and women.

Expression of *miR-1*, *miR-133a/b, and miR-208a* in fetal hearts and children compared to healthy adults

Using the Kruskal-Wallis test, the expression of miRNAs *miR-1, miR-133a/b,* and *miR-208a* was analyzed for statistical significant differences between the three groups (fetal hearts, children, and healthy adults). The expression of *miR-133a* and *miR-133b* was found to be significantly different between the three groups ($P = 0.000$ and $P = 0.019$, respectively).

Further analysis using the Mann-Whitney test showed statistically significant down-regulation of *miR-133a* and *miR-133b* in fetal hearts as well as in children when compared to healthy adults. The down-regulation of *miR-133a* reached ~6-fold in fetal hearts ($P = 0.000$) and ~4.3-fold in children ($P = 0.002$), whereas down-regulation of *miR-133b* was ~3.3-fold in fetal hearts ($P = 0.006$) and ~3-fold in

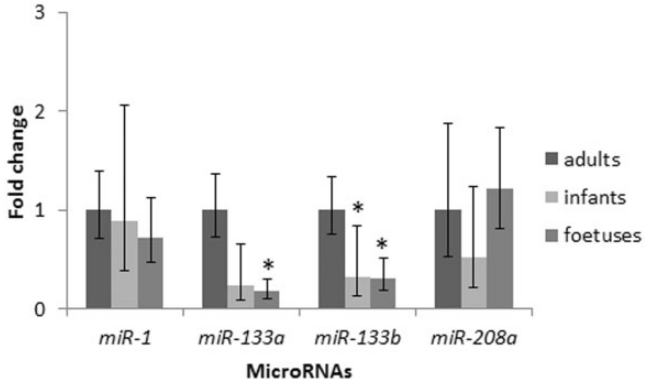

Figure 1 Expression of *miR-1*, *miR-133a/b,* and *miR-208a* in human fetal hearts compared to expression in the hearts of children and healthy adults. *Statistically significant change in expression; *n*, number of samples; $n_{\text{fetal hearts}} = 45$; $n_{\text{children}} = 13$; $n_{\text{healthy adults}} = 15$

children ($P = 0.038$). Mann-Whitney test confirmed that there was no statistical difference in the expression pattern of *miR-1* and *miR-208a* between fetal hearts and healthy adults or between children and healthy adults. There was also no statistical difference in the expression of *miR-1, miR-133a/b,* or *miR-208a* between fetal hearts and children. Results are summarized in Figure 1.

We further tested how analyzed miRNAs in fetal hearts, children, and healthy adults are expressed in correlation to each other. We found that *miR-133b* was in a statistically significant positive correlation with *miR-1* ($r_s = 0.631$, $P = 0.000$), *miR-133a* ($r_s = 0.857$, $P = 0.000$) and *miR-208a* ($r_s = 0.409$, $P = 0.003$). The same was true for *miR-1,* which was in a statistically significant positive correlation with *miR-133b*, *miR-133a* ($r_s = 0.507$, $P = 0.000$) and *miR-208a* ($r_s = 0.437$, $P = 0.001$). However, there was no correlation observed between the expression of *miR-208a* and *miR-133a*.

Expression of *miR-1*, *miR-133a/b,* and *miR-208a* in fetal hearts of different gestational ages

We further tested the hypothesis that there is correlation between the expressions of the miRNAs analyzed and gestational age. We found that *miR-208a* is expressed in negative correlation to gestational age ($r_s = -0.356$, $P = 0.028$), showing a decrease in *miR-208a* expression with increasing gestational age. Results are summarized in Figure 2a.

We found that when analyzing fetal hearts alone, *miR-133b* is again expressed in a statistically significant correlation with *miR-1* ($r_s = 0.354$, $P = 0.029$), *miR-133a* ($r_s = 0.589$, $P = 0.000$), and *miR-208a* ($r_s = 0.368$, $P = 0.023$). All other miRNAs, including the expression of *miR-1* and *miR-133a,* showed no correlation to each other in fetal hearts. Results are summarized in Figure 2b.

Expression of cleaved-CK18, and its correlation to the expression of *miR-1*, *miR-133a/b,* and *miR-208a*

As previously described, cleaved-CK18 positive cells were found in all fetal hearts.[17] Their density was determined by a morphometric approach. Comparing cleaved-CK18 staining from different gestational ages, we observed a significant decrease in expression from 25 th to 29 th gestational weeks. The density of cleaved-CK18 positive cells ranged from 0.7 to 16.1 per mm^2 and it decreased with increasing gestational age. In infants below the age of 6 months, the density of cleaved-CK18 positive cells ranged from 1.78 to 2.14 per mm^2 in the first postnatal month and decreased to 0.35 per mm^2 in 11 postnatal months. In children older than 16 months no positive cardiomyocyte was observed. However, no immunostaining was seen in the cardiomyocytes from adult hearts. Photographs of apoptotic cells (as identified by immunostaining with cleaved-CK18) are represented in Figure 3a.

In this study, we also analyzed whether there is any correlation between the expression of cleaved-CK18 as a marker of apoptosis, and the expression of miR-*1*, *miR-133a/b,* and *miR-208a*. We found that the expression of *miR-133a* and *miR-133b* in children and fetal hearts is in a statistically significant negative correlation with the expression of cleaved-CK18 ($r_s = -0.437$, $P = 0.026$ and $r_s = -0.517$, $P = 0.007$, respectively). Results are summarized in Figure 4a. Solely considering fetal hearts, only *miR-133b* is in a statistically significant negative correlation with the expression of cleaved-CK18 ($r_s = -0.426$, $P = 0.036$). Results are summarized in Figure 4b. The expression of *miR-1* and *miR-208a* did not show any statistically significant correlation to the expression of cleaved-CK18.

Expression of the proliferative marker, Ki67, in different gestational ages

Ki67-positive cells were found in all fetal hearts. The density of positive cells was determined by a morphometric approach. No immunostaining was seen in the cardiomyocytes from the 15 adult hearts.

We analyzed the expression of Ki67 in fetal hearts of different gestational ages, and observed extremely high proliferative activity in the second quarter of fetal life (13 th–20 th gestational weeks), when the density of positive cells ranged from 410 to 747 per mm^2. There was a significant decrease in the expression of Ki67 from the 13th to 41st gestational weeks; with the most significant decrease observed after 31 st gestational weeks when the density achieved was only 31–37 cells/mm^2, and was 1.7–7.5 cells/mm^2 after the 38 th gestational week. The density of Ki67-positive cells in neonates was even slightly elevated (5.3–7.1 per mm^2) after the age of three months, decreasing again to around 0.71 per mm^2 in the first year of age. After the 14 months of age Ki-67 expression was no further detectable. We therefore analyzed the correlation between the expression of the proliferation marker Ki67 and gestational age, and found that there is a statistically significant negative correlation ($r_s = -0.937$, $P = 0.000$). Photographs of proliferative cells (as identified by immunostaining with Ki67) are represented in Figure 3b.

Correlation between the expression of Ki67 and *miR-1*, *miR-133a/b,* and *miR-208a* at different gestational ages

Next, we tested whether there is any correlation between the expression of Ki67 and the analyzed miRNAs in fetal,

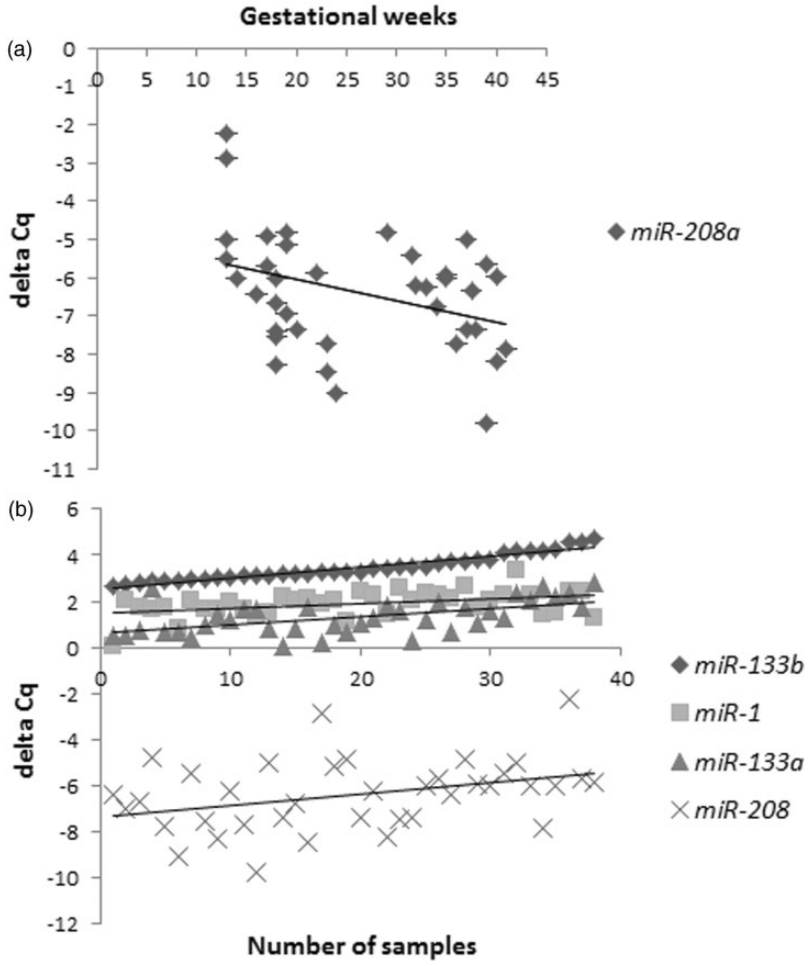

Figure 2 Expression of miRNAs in fetal hearts. a, *miR-208a* in correlation with gestational age; b, miR-133b in correlation to *miR-1*, *miR-133a,* and *miR-208a*.*n*, number of samples; $n_{\text{fetal hearts}} = 45$; trend line has been added to outline positive or negative correlation

children and adult hearts. Considering all of the samples, we observed a statistically significant negative correlation between the expression of Ki67 and the expression of *miR-133a* ($r_s = -0.352$, $P = 0.033$) and *miR-133b* ($r_s = -0.331$, $P = 0.046$). We further compared the expression of miRNAs and the expression of the proliferation marker Ki67 in the fetal hearts. There was a statistically significant positive correlation between the expression of Ki67 and *miR-208a* ($r_s = 0.381$, $P = 0.045$). Results are summarized in Figure 5a.

Since there was a significant decrease in Ki67 staining after the 29th gestational week, we further divided the fetal hearts in two groups, from the 13 th to 25 th gestational week (24 samples) and from the 29 th to 41 st gestational week (22 samples). We compared the expression of all four miRNAs, *miR-1, miR-133a/b*, and *miR-208a* with the expression of Ki67. We found that the expression of *miR-208a* is in a statistically significant positive correlation with the expression of Ki67 before the 25 th gestational week ($r_s = 0.605$, $P = 0.022$). After the 25 th gestational week, expression of both, *miR-208a* ($r_s = 0.530$, $P = 0.05$) and of *miR-1* ($r_s = 0.531$, $P = 0.05$), is in a weak, but significantly positive correlation with the expression of Ki67. However, there was no statistically significant correlation between the expression of *miR-133a/b* and Ki67 density. Results are summarized in Figure 5b.

Discussion

In this study, we observed a significant down-regulation of *miR-133a* and *miR-133b* in fetal hearts and children compared to healthy adults, and a progressive but non-significant up-regulation of *miR-1* from fetal to children and to healthy adult hearts. We have not found any significant difference in the expression of *miR-208a* in fetal hearts or children when compared to healthy adults. There was also no significant change in the expression of *miR-1, miR-133a/b,* and *miR-208a* in fetal hearts compared to children. However, a significant decrease in *miR-208a* expression with increasing gestational age was observed. We also observed a significant positive correlation between the expression of *miR-1* and *miR-133b* as well as a significant positive correlation of both miRNAs with the expression of *miR-133a* and *miR-208a*. Some of these results are in accordance to our previous study, which has now been confirmed in a larger cohort of fetal samples.[11] It has been consistently shown that *miR-1* and *miR-133* are expressed in very small amounts in developing mouse and zebrafish hearts, where

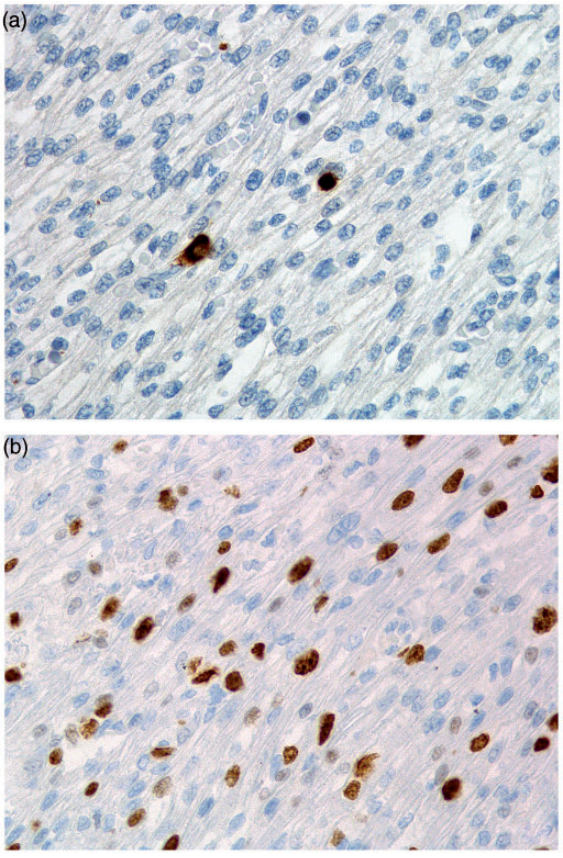

Figure 3 Immunohistochemical expression of markers of apoptosis and proliferation. a, cleaved-CK18 as marker of apoptotic cells in the fetal hearts (15 weeks of gestation), recognized by antibody M30; b, Ki67 as a marker of proliferating cells in the fetal hearts (15 weeks of gestation), as detected by antibody MIB-1. (A color version of this figure is available in the online journal.)

increasing expression was found in neonatal hearts, but still at substantially lower levels than that in adult tissues.[21,22] According to previously published data, *miR-208a* was specifically detected in heart of the adult mouse, and could be detected at very low levels in the heart as early as the embryonic stage.[14] It has been also shown that alpha-MHC, encoding *miR-208a*, decreases with increasing gestational age in developing human hearts and that the adult level of alpha-MHC, when established during fetal development, does not subsequently change.[23] The results of our study thus further support the suggestion that the expression of *miR-208a* in the human fetal hearts is reflecting the expression of its host gene, alpha-MHC, observed otherwise in a study using experimental animals.[14] It appears that all four miRNAs, cardiac and/or muscle-specific, in the human heart are under similar transcriptional or expression regulation throughout the life cycle which might vary at different points during development.

We found that the expression of *miR-133a/b* is in significant negative correlation with apoptosis (detected by the expression of cleaved-CK18) in the fetal hearts and in children. We also showed that *miR-133b* is in negative correlation with apoptosis in the fetal hearts. Both of these results suggest that *miR-133a/b* has an anti-apoptotic function. Although *miR-1* and *miR-133*a have been suggested to have opposite roles in apoptosis and are part of a bicistronic unit, we did not observe *miR-1* correlating with the apoptotic rate in fetal hearts or in children. This may be explained by the presence of putative TF binding sites between *miR-1* and *miR-133a*, suggesting the possibility that the individual miRNAs contained in the polycistronic unit may be independently regulated.[22] In our previous study, we observed that apoptosis, also measured by cleaved-CK18, in the fetal human heart resembles apoptosis in epithelial tissue and that it decreases with increasing gestational age.[17] Apoptosis is an essential physiological process which plays a critical role in controlling the number of cells in development and removing cells at the appropriate time.[24] It has been shown recently that in hypoxia-induced apoptosis of neonatal cardiomyocytes, the administration of tanshinone IIA significantly reduced the rate of apoptosis and up-regulated the expression of *miR-133* through the MAP ERK1/2 signaling pathway. These results also suggest an anti-apoptotic function of *miR-133*.[25] Accordingly, the compound loss of both copies of *miR-133a* in mice (*miR-133a-1* and *miR-133a-2*) resulted in enhanced apoptosis.[9,26]

Finally, we observed a decreasing proliferation index with increasing gestational age, as measured by the expression of Ki67, where proliferation rates remained high until the 25 th gestational week, but decreased significantly after the 29 th gestational week. Considering all of the heart samples, including fetuses, children, and healthy adults, we found that the expression of *miR-133a* and *miR-133b* is in a significant negative correlation with proliferation, suggesting their anti-proliferative function. Our results for *miR-133* expression are in accordance to those using experimental mice, suggesting an inhibitory function of *miR-133a* on cardiomyocyte proliferation.[26] While *miR-133a-1* and *miR-133a-2* do not seem to cause obvious cardiac abnormalities when deleted individually, the targeted deletion of both miRNAs results in late embryonic or neonatal lethality and cardiac malformations. The absence of *miR-133a* resulted in the ectopic expression of smooth muscle genes in the heart and in an enhanced cardiomyocyte proliferation throughout the ventricles, targeting cyclin D2.[4,16,26] Overexpression of *miR-133a* resulted in a phenotype opposite to that of *miR-133a* deletion, being consistent with the conclusion that *miR-133a* suppresses cardiomyocyte proliferation.[26] *miR-133* also regulates cardiac proliferation by inhibiting RhoA and Cdc42. Progressive down-regulation of the proteins of the actin-myosin ring (RhoA, Cdc42) has an impact on decreasing the proliferative activity of cardiomyocytes, which is in correlation with increasing gestation.[27,28]

Only considering fetal hearts, there is a significant positive correlation between the expression of *miR-208a* and Ki67, and a weakly significant positive correlation of *miR-1* and Ki67 after the 25 th gestational week, suggesting their proliferative function. Similar results concerning proliferation in fetal hearts by using Ki67 immunostaining have already been described.[29] A recently proposed role for *miR-208* in promoting insulin-induced vascular smooth muscle cell proliferation and our results suggest that *miR-208a* may

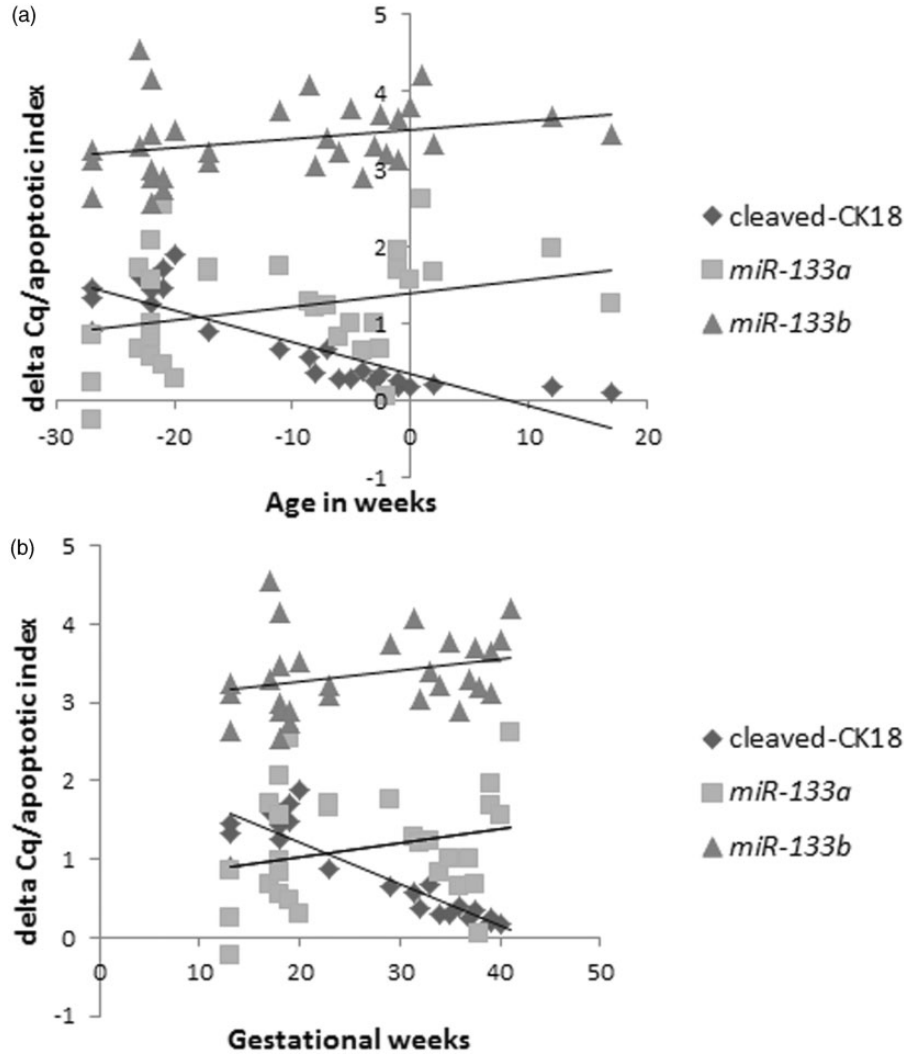

Figure 4 Expression of *miR-133a/b* in correlation with the expression of cleaved-CK18. a, expression in fetal hearts and children; b, expression in fetal hearts. Apoptotic index is divided by factor 10 for better representation of correlation to miRNAs expression; trend line has been added to outline negative correlation; a, for better representation of expression patterns before and after birth, birth is set at 0 and all other ages are calculated in negative or positive weeks (fetal hearts are in negative weeks and children in positive weeks), *n*, number of samples; $n_{\text{fetal hearts}} = 45$; $n_{\text{children}} = 7$

also have a similar function in the developing human heart.[30]

Several studies have showed that cardiac-specific overexpression of *miR-1* in the embryonic heart inhibits cardiomyocyte proliferation (an effect in opposition to our observations) by targeting Hand2, a protein involved in myocyte expansion, and promotes muscle differentiation.[2,10,14,15] However, it should be mentioned that the majority of these studies were performed on cell lines or animal embryos, and our correlation was observed later in fetal life. In *miR-1-2* knock-out mice, with a deletion of one of the two copies of *miR-1*, the mutant mice displayed an unusual presence of mitotic adult cardiomyocytes in addition to wide range of abnormalities in the embryo (e.g. ventricular septal defects), including hyperplasia of the heart with nuclear division persisting post-natally in the mice that survive until that point.[2] However, this was observed when deleting only one of the two copies of *miR-1* within the genome. The question remains of what happens when only the second copy (*miR-1-1*) is missing, or whether deletion of both copies evokes a more severe phenotype. Although having the same mature sequences, the two copies of *miR-1* seem to have non-redundant roles and slightly different spatial expression patterns; while *miR-1-1* is initially strongly expressed in the atria, *miR-1-2* can be found mainly in the ventricles.[31] However, expression on whole sections of fetal heart was performed in our study, including both the atria and the ventricles. Furthermore, using qPCR for measuring *miR-1* expression, as in our study, could not distinguish between two copies; therefore only the overall or absolute concentration was analyzed, independent of the *miR-1-1* and *miR-1-2* ratio.

In conclusion, in this study we showed that the cardiac expression of *miR-1* and *miR-133a/b* in fetal hearts is similar to its expression in children, but different from that in healthy adults. We also found that expression of *miR-208a* in fetal hearts correlates with gestational age. Our results also show that *miR-1, miR-133a/b,* and *miR-208a* correlate

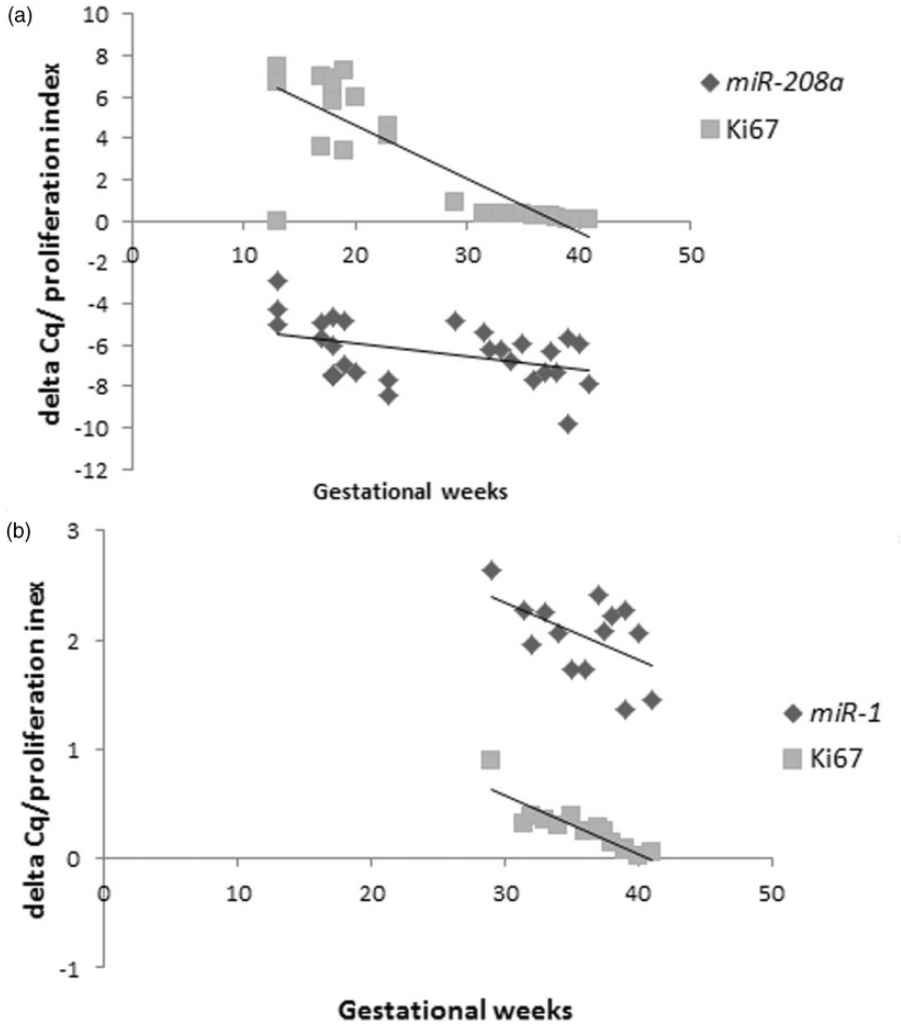

Figure 5 Expression of Ki67, *miR-208*, and *miR-1* in correlation with gestational age and with each other. a, Expression of *miR-208a* in correlation to the expression of Ki67 in fetal hearts of different gestational weeks; c, expression of *miR-1* in correlation to the expression of Ki67 in fetal hearts from the 29 th to the 41 st gestational weeks. Proliferation index is for better representation of correlation divided by factor 100; *n*, number of samples; $n_{\text{fetal hearts}} = 45$

with the proliferation of cardiomyocytes throughout the life cycle. These results, and the fact that *miR-1* and *miR-133a/b* expression in children is similar to that in fetal hearts but different from that in adults, also support the suggestion that cardiomyocytes retain some proliferative capacity in the postnatal period as previously described.[29] Finally, we confirmed the suggestion that *miR-133a/b* may have anti-apoptotic activity in fetal hearts.

Author contributions: EB and MJ contributed equally to this work. All authors participated in the design, interpretation of the study, analysis of the data, and the review of the manuscript: EB conducted the experiments, EB and MJ analyzed the data and wrote the article, DG and NZ supervised the study and critically revised the article.

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