Mol Cell Biochem (2009) 324:1–11
DOI 10.1007/s11010-008-9974-3

Cell type-specific expression of endogenous cardiac Troponin I antisense RNA in the neonatal rat heart

Stefanie Voigtsberger · Holger Bartsch · Gert Baumann · Hans Peter Luther

Received: 30 June 2008 / Accepted: 28 November 2008 / Published online: 30 January 2009

Abstract Since the number of detected natural antisense RNA is growing, investigations upon the expression pattern of the antisense RNA become more important. As we focused our work on natural occurring antisense transcripts in human and rat heart tissues, we were interested in the question, whether the expression pattern of antisense and sense RNA can vary in different cell types of the same tissue. In our previous analysis of total neonatal rat heart tissue, we demonstrated the co-expression of both cTnI RNA species in this tissue. Now we investigated the expression of antisense and sense RNA quantitatively in neonatal cardiomyocytes (NCMs) and neonatal cardiac fibroblasts (NCFs). Performing northern blot as well as RT-PCR, we could detect natural antisense and sense RNA transcripts of cTnI in NCM and NCF implying that these transcripts are co-expressed in both cell types. The absolute amounts of the RNA transcripts were higher in NCM. Both RNA species showed identical sizes in the northern blot. Quantification by real-time PCR revealed a higher relative level of natural antisense RNA in NCF compared to NCM which points out to a cell type-specific expression of sense and antisense RNA. Our observations suggest that antisense RNA transcription may contribute to a cell type-specific regulation of the cTnI gene.

Keywords Natural antisense RNA · Gene regulation · Cardiac Troponin I · Neonatal cardiomyocytes · Neonatal cardiac fibroblasts

S. Voigtsberger · G. Baumann
Medizinische Klinik mit Schwerpunkt Kardiologie und Angiologie, Charité—Universitätsmedizin Berlin, CCM, Charitéplatz 1, 10117 Berlin, Germany

H. Bartsch
Institut für Immunologie, Medizinische Fakultät Carl Gustav Carus, Technische Universität Dresden, Fiedlerstrasse 42, 01307 Dresden, Germany

H. P. Luther (✉)
Departement für Innere Medizin, Inselspital, Medizinische Fakultät der Universität Bern, 3010 Bern, Switzerland
e-mail: hanspeter_luther@yahoo.de

Introduction

Over the last few years, a growing number of natural antisense RNA transcripts of eukaryotic genes have been detected. It is now widely accepted that antisense transcripts are involved in the regulation of at least 10–20% of human genes [1]. Therefore, antisense transcription appears to be a relevant principle of gene regulation in mammalian cells [1].

However, the exact mechanisms of natural antisense RNA regulation are still discussed controversially. Likewise, the detected antisense transcripts of different genes show a strong heterogeneity. So far a consistent model of the origin, structure, and function of natural antisense RNA has not been established.

Our group has focused on the detection of natural antisense RNA of contractile proteins from cardiac tissue. In previous studies, natural occurring antisense RNA of α and β Myosin Heavy Chain (MyHC) have been detected and characterized in rat heart tissue [2, 3]. Moreover, a co-regulation of sense and antisense RNA for α-MyHC and for β-MyHC under stimulation with the thyroid hormone T3 (L-trijodthyronin) and the potent α_1-agonist phenylephrine was shown in neonatal cardiomyocytes (NCMs) [4].

Additionally, the antisense RNA expression of cardiac Troponin I (cTnI) was investigated. Cardiac Troponin I, as a part of the heterotrimeric troponin complex consisting of

Table 1 Sequence and characteristics of the used primers

Primer	Sequence (5′–3′)	Orientation	Used for
TnI rat F20	TCA CCA GGG ACA CCC TTC TAA	Forward	RT
TnI rat F255	ATT GCG AAG CAG GAG ATG GAG CGT GAG	Forward	PCR
TnI rat F311	GCG TTC TGA GCA CGC GTT GC	Forward	RT
TnI rat F509	AGC GGC CCA CTC TCC GAA GA	Forward	Real-time PCR
TnI rat R559	CCT GCA TCA TGG CAT CTG CTG A	Reverse	PCR, Real-time PCR
TnI rat R731	CCT CCA GGA TCC GCC ATG GCT CAG CCC TCA AAC	Reverse	RT, PCR
F-GAPDH	GCA TCG TGG AAG GGC TCA T	Forward	RT, PCR
R-GAPDH	GAT GGG GAC TCC TCA GCA AC	Reverse	RT, PCR
F-HPRT	CTT GCT CGA GAT GTC ATG AAG G	Forward	RT, Real-time PCR
R-HPRT	AAT CCA GCA GGT CAG CAA AGA	Reverse	RT, Real-time PCR

cTnI, Troponin T, and Troponin C, plays an essential role in the normal contraction cycle by regulating conformational changes in the Troponin/Tropomyosin complex [5, 6]. The existence of antisense-orientated RNA transcripts of cardiac-specific Troponin I was demonstrated in rat and human myocardium [7]. To date, antisense transcripts are thought to form duplexes with their complementary sense strand, and thereby regulate gene expression at a post-transcriptional level [4]. Previously, we could demonstrate such an in vivo duplex formation between antisense and sense transcripts of cTnI in cardiomyocytes [7]. Moreover, a potential effect of attenuating translation by natural antisense transcripts was shown by in vitro transcription/translation experiments with synthetic antisense oligonucleotides (ASO) directed against the translation initiation site of mRNA. In addition, a significant reduction of cTnI protein level upon treatment with these ASO was demonstrated in cultured cardiomyocytes [7].

Investigating the molecular structure of these antisense transcripts by applying RACE-PCR, a complete homology between the antisense RNA sequence and the published cTnI mRNA sequence has been demonstrated recently [8]. Therefore, we hypothesized that cTnI antisense RNA is transcribed from the sense mRNA in the cytoplasm, which is in accordance to data from systematic identification of antisense transcripts by Røsok and Sioud [9]. Moreover, a RNA-hybrid structure was detected comprising sense and antisense in one continuous molecule. It was concluded that this RNA-hybrid is a result of the close interaction of sense and antisense RNA in vivo [8].

Up to now, cTnI is primarily known as a muscle-specific protein that plays a crucial role in contraction regulation in the myocardium and that is expressed in cardiomyocytes exclusively [6]. However, muscle-specific proteins have already been detected in non-muscle cells [10]. Regarding Troponin I, it has also been reported that the fast-skeletal isoform is expressed in non-muscle tissue like human cartilage where it can act as a potent and specific inhibitor of angiogenesis in vivo and in vitro, as well as of tumor metastasis in vivo [11].

In the normal heart, two-thirds of the cell population is composed of non-muscle cells, especially fibroblasts. Recent studies have shown that cardiac fibroblasts play an essential role in cardiac remodeling [12].

To date, we have focused on the detection of natural antisense RNA in total cardiac tissue or isolated cardiomyocytes. Quantification revealed equal amounts of antisense and sense transcripts in total cardiac tissue.

There are strong evidences that antisense and sense RNA of a specific gene can be expressed in a specific cell type although the corresponding protein is not expressed. This was previously shown for the endothelial NO-synthetase (eNOS) in vascular smooth muscle cells [13]. In this case, it was speculated that antisense RNA expression is involved in gene-specific down-regulation of the translation product.

The aim of the present study was to investigate the cell type-specific expression of antisense and sense RNA of cTnI in neonatal cardiac fibroblasts (NCFs) in comparison to NCMs to elucidate cell type-specific antisense RNA transcription in the two main cell populations of the myocardium.

Results

Verification of the culture conditions

As a prerequisite for the following investigations, the purity of the cultured NCF and NCM was an essential aspect and was confirmed in all cases by morphological examinations after staining the cells with Phalloidin-FITC and DAPI (Fig. 1). NCF are thin, triangular-formed cells with a lighter cytoplasm (Fig. 1, (1) 10-fold magnification and (3) 5-fold magnification). NCMs show a more extended surface and a more intense staining of the cytoplasm

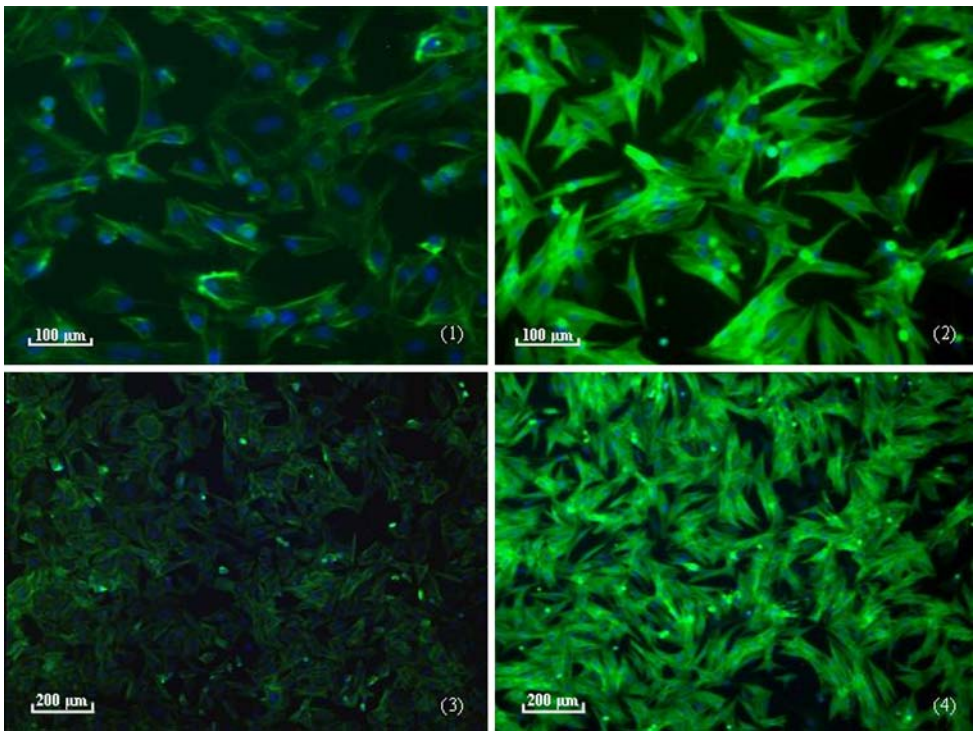

Fig. 1 Phalloidin assay of neonatal cardiac fibroblasts (NCFs) (**1**, **3**) and neonatal cardiomyocytes (NCMs) (**2**, **4**). NCF (short-term culture, 5 days of culture) at 10-fold (**1**) and 5-fold (**3**) magnification are thin triangular formed cells with a light cytoplasm whereas NCM (primary culture, 5 days of culture) at 10-fold (**2**) and 5-fold (**4**) magnification are characterized by a more intense staining of the contractile filaments in the cytoplasm

caused by the contractile filaments (Fig. 1, (2) 10-fold magnification and (4) 5-fold magnification). The results could demonstrate that the culture conditions used herein are sufficient to discriminate between NCM and NCF yielding a purity of nearly 99%.

Detection of cTnI antisense RNA by RT-PCR

Performing RT-PCR with two different cTnI-specific primer pairs (F255/R731 and F255/R559), we could detect antisense- and sense-specific transcripts in NCF from passage 3 (short-term culture, 5 days of culture) (Fig. 2) as well as in NCM (primary culture, 5 days of culture) (Fig. 2).

Equal amounts of RNA were used for strand-specific reverse transcription. In all cases, the integrity of the cDNA was tested by amplification of a house keeping gene (GAPDH) as a control. For the reverse transcription with sense- or antisense-specific cTnI primer, a GAPDH-specific primer was used in the same RT experiment. In all cases, the expected GAPDH fragment was detectable.

RT-PCR resulted in the expected fragment size (476 bp for primer F255/R731 and 304 bp for F255/R559). In all cases, reverse transcription with unspecific oligo dT primer resulted in a more intense signal then the sense- or antisense-specific reverse transcription. Also, the fragment intensity obtained in the antisense-specific RT-PCR gave the weakest signal. As both primer pairs were located in different exons, amplification of genomic DNA could be ruled out.

Detection of cTnI antisense RNA by northern blot

The northern blot analysis also revealed the existence of both antisense and sense transcripts in NCF from passage 3 (short-term culture, 5 days of culture) and in NCM (primary culture, 5 days of culture) (Fig. 3(1)). Both antisense and sense transcripts showed identical sizes (800 bp) in both cell types. This was confirmed by parallel non-radioactive detection of the positive controls of either antisense (700 bp) or sense synthetic RNA (500 bp) generated by in vitro transcription. Total RNA of NCF from passage 1 (2 days of culture) was parallel loaded on the gel. The expression of antisense and sense transcripts was also shown in these cells. However, one cannot exclude that the cTnI-specific RNA signal in this probe is caused by a contamination of NCM. This can be ruled out in NCF from passage 3 since we could show that NCF after three passages have a purity of nearly 99% when the outlined protocol is applied.

Our data confirm that the expression of antisense and sense RNA in NCF of passage 3 (short-term culture, 5 days of culture) is comparable with those of NCF at passage 1 (2 days of culture). NCM (primary culture, 5 days of culture) showed a higher expression of sense and antisense RNA in all experiments.

Northern blot analysis with NCF from passage 4 (long-term culture, 14 days of culture) revealed that the expression of cTnI sense and antisense RNA decreases rapidly in longer cultured fibroblasts suggesting a possible process of down-regulation of cTnI sense and antisense

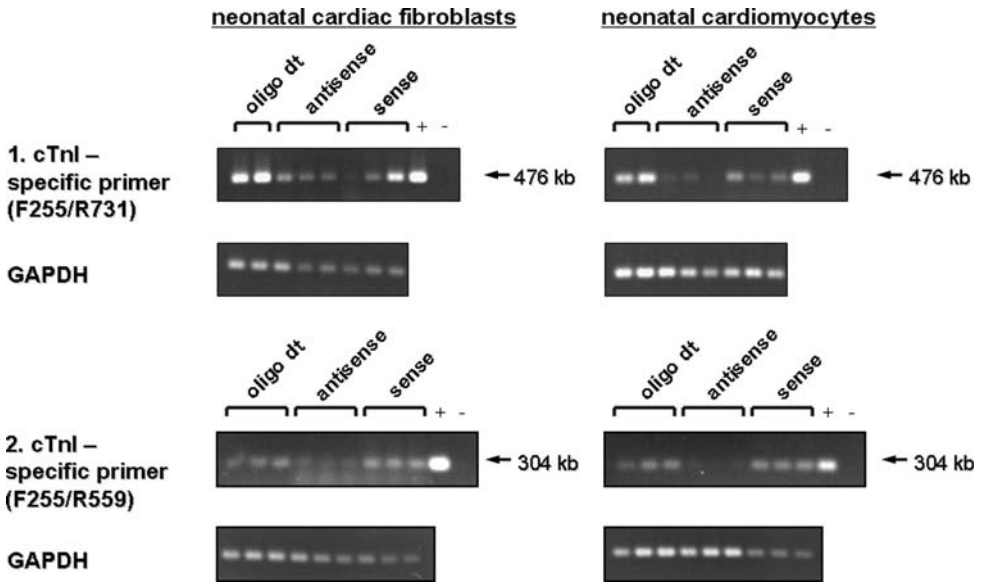

Fig. 2 Detection of cTnI antisense and sense RNA by strand-specific RT-PCR. Strand-specific RT-PCR with cTnI-specific primer pairs F255/R731, F255/R559 and GAPDH (housekeeping gene). PCR was performed with 500 ng cDNA generated by specific reverse transcription using the primer F20 (antisense-specific), R731 (sense-specific) or oligo dt (unspecific). Three lines of both antisense and sense are shown, representing three different reverse transcriptions with three different RNA probes, except for the unspecific oligo dt transcription in the case of F255/R731, where only two different reverse transcription experiments are shown. Specific cTnI products with expected sizes (476, 304 kb as indicated by arrows) were detected in NCF (passage 3, short-term culture) and NCM. RT-PCR of GAPDH as a housekeeping gene showed the expected signal in all RNA probes

RNA due to culture condition (mono-culture) and culture time (Fig. 3(2)).

Equal amounts of isolated total RNA (up to 20 μg) were loaded on the gel in all cases. The equable transfer of total RNA onto the blot was confirmed by staining with an unspecific dye (bromphenol-blue) after the detection of the RNA (data not shown). The transfer was equal in all cases which allowed the quantitative comparison of isolated total RNA in both cell types.

Quantification of cTnI antisense RNA by real-time PCR

Since an exact quantification of the RNA was not possible in the previous experiments, we applied real-time PCR. The cTnI-specific primer pair F509/R559 and the housekeeping gene HPRT were used. The expected fragment sizes of 50 bp for cTnI and 75 bp for HPRT were detectable after the separation of the PCR product on a 5% polyacrylamid gel. No further PCR fragments were visible, indicating the specificity of the PCR conditions used (data not shown).

The ΔC_t-values were calculated by normalization of the $C_{t_{cTnI}}$-value against the $C_{t_{HPRT}}$-value. This resulted in data without units which represent the relative expression of cTnI normalized to HPRT.

Real-time PCR revealed a 5-fold higher expression of antisense RNA (14.6 ± 5.5 vs. 2.8 ± 0.5; $P < 0.05$) and a 26-fold higher expression of sense RNA (967 ± 315 vs. 36 ± 9; $P < 0.01$) in NCM compared with NCF from passage 3 (short-time-culture, 5 days of culture) (Fig. 4). These data confirm the results of the northern blot that showed a higher expression of both sense and antisense RNA in NCM.

In addition, we compared antisense/sense ratios in NCF at different passages (passage 3, short-term culture and passage 4, long-term culture) and NCM (primary culture) to determine the cell type-specific relative expression of antisense RNA. Therefore, the antisense/sense ratios were calculated by division of normalized C_t-values of the antisense product by normalized C_t-values of the sense product for each real-time PCR experiment. Our analysis revealed a significant higher relative antisense RNA expression in NCF from passage 4 (long-term culture, 14 days of culture) compared to NCM (primary culture, 5 days of culture) (0.13 ± 0.02 vs. 0.05 ± 0.02; $P < 0.05$) (Fig. 5 (1)). Simultaneously, this result shows that sense RNA is in an enormous excess in NCM (66-fold) and also in NCF (12-fold). In addition, we compared the different passages of NCF (passage 3, short-term culture and passage 4, long-term culture). There were no significant differences in antisense/sense ratios between the different passages of NCF (0.13 ± 0.02 vs. 0.11 ± 0.02) (Fig. 5 (2)). In conclusion, this result shows that the antisense/sense ratio is higher in NCF compared to NCM independent from culture time and passage and therefore indicates a cell type-specific expression pattern of antisense and sense RNA.

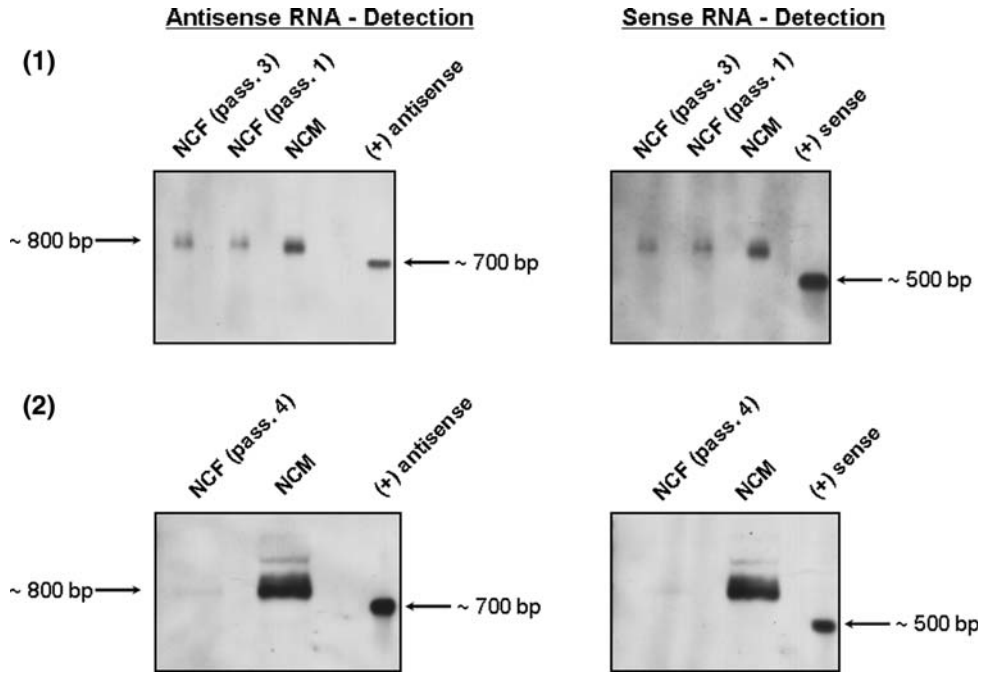

Fig. 3 Detection of cTnI antisense and sense RNA by Northern Blot. (**1**) About 10 μg of total RNA isolated from NCF (passage 3) (short-term culture, 5 days of culture), NCF (passage 1) (2 days of culture) and from NCMs (primary culture, 5 days of culture) were hybridized first with 100 ng/μl *antisense-specific* DIG-labeled cTnI RNA (pSPT19 cTnI) overnight. After detection and stripping, the same blot was hybridized with 100 ng/μl *sense-specific* DIG-labeled cTnI RNA (pSPT18 cTnI). As positive control in vitro transcribed unlabeled cTnI RNA with known length (~700 bp for cTnI antisense RNA, ~500 bp for cTnI sense RNA) revealed the specificity of the detection. Specific signals for antisense and sense RNA with the expected size of ~800 bp were detected in NCF as well as in NCM. Antisense and sense RNA signals were more intense in NCM. (**2**) About 20 μg of total RNA isolated from NCF (passage 4) (long-term culture, 14 days of culture) and NCM (primary culture, 5 days of culture) were detected with the same antisense- and sense-specific RNA probes. Again specific signals for antisense and sense RNA were detectable in NCM. Signal intensity was decreased impressively in NCF from long-term culture

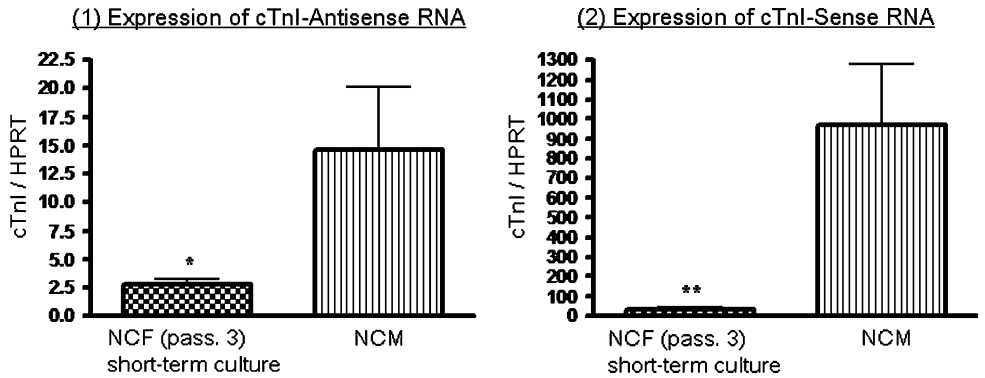

Fig. 4 Quantification by real-time PCR: comparison of cTnI antisense RNA expression (**1**) and cTnI sense RNA expression (**2**). Real-time PCR revealed a 5-fold higher expression of antisense RNA (**1**) and a 26-fold higher expression of sense RNA in NCM (primary culture, 5 days of culture) compared to NCF (passage 3) (short-term culture, 5 days of culture). Values are based on data from $n = 3$ and expressed as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$

Detection of cTnI protein expression by western blot

Western blot analysis were performed with protein samples extracted from NCM (primary culture, 5 days of culture) and NCF (long-term culture, 14 days of culture) to elucidate a possible role of endogenous antisense RNA on cTnI expression and to verify the culture conditions. Immunodetection with a cTnI-specific antibody resulted in the expected signal at ~20 kDa in NCM, whereas no cTnI signal was detectable in NCF (Fig. 6). This result is of particular importance because the RNA expression studies showed higher absolute amounts of antisense RNA expression in

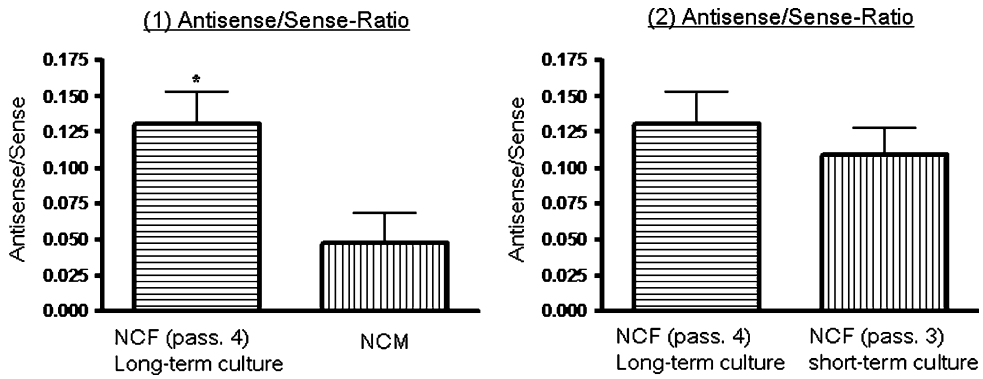

Fig. 5 Quantification by real-time PCR: comparison of the antisense/sense ratio. Antisense/sense ratios were calculated by division of normalized C_t-values of the antisense product by normalized C_t-values of the sense product for each real-time PCR experiment. A higher value represents a higher relative expression of antisense RNA. (**1**) A significant higher relative antisense RNA expression was observed in NCF (passage 4) (long-term culture, 14 days of culture) compared to NCM (primary culture, 5 days of culture) (0.13 ± 0.02 vs. 0.05 ± 0.02, $^*P < 0.05$]. (**2**) Comparing the different passages of NCF (passage 3, short-term culture and passage 4, long-term culture) no significant differences in antisense/sense ratios were detectable (0.13 ± 0.02 vs. 0.11 ± 0.02). Values are based on data from $n = 3$ and expressed as means ± SEM

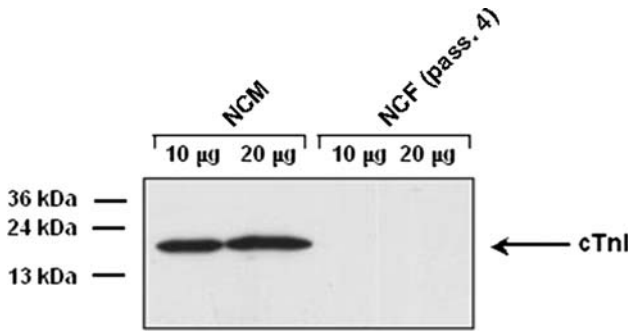

Fig. 6 Detection of cTnI protein by western blot. About 10 and 20 µg of total cell protein isolated from NCM (primary culture, 5 days of culture) and NCF (long-term culture, 14 days of culture) were separated by SDS-PAGE followed by western blotting. Detection by a specific cTnI antibody showed the expected signal for cTnI in NCM, whereas no cTnI signal was detectable in NCF

NCM. So, despite the high absolute antisense RNA expression in NCM cTnI protein is expressed.

Discussion

In the present study, we investigated the expression pattern of sense and antisense RNA of cTnI in NCMs and NCFs.

Our investigations could clearly proof the existence of sense and antisense RNA of cardiac Troponin I in NCM as well as in NCF by conventional RT-PCR and by northern blot. Both transcripts had the expected size of 800 bp. This result is in accordance to the previous northern blot analysis of total rat myocardium where we could also detect both RNA transcripts with the expected size of the spliced mRNA [7].

Furthermore, we applied real-time PCR for the accurate quantification of the expression of antisense and sense RNA. Real-time PCR analysis revealed a higher absolute expression of antisense and sense RNA in NCM and therefore confirmed the northern blot data.

The question of co-expression of antisense and sense RNA in one tissue or cell type is discussed controversially. Spencer et al. detected specific natural antisense and sense RNA transcripts of the DOPA-decarboxylase in Drosophila. These transcripts were never expressed in parallel in one tissue but their expressions were mutually exclusive [14]. However, there are other reports referring to a co-expression of natural sense and antisense transcripts of a gene in one tissue, for example, urocortin which is a well-characterized CRF-related neuro-peptid [15].

The fact of co-expression of antisense and sense transcripts of cTnI is in accordance to our previous findings of in vivo duplex formation of sense and antisense RNA in cardiomyocytes, because this formation can only take place when both transcripts are expressed in one cell type [7]. Moreover, a recent publication by Chen et al. points out that simultaneous expression of sense and natural occurring antisense RNA is an essential step and an important indicator for in vivo antisense regulation. The estimated amount of human genes that form sense/antisense pairs is ~20% [16]. Therefore, it is reasonable to conclude that the co-expression of cTnI antisense and sense RNA in NCM and NCF is an indicator of cTnI gene regulation by natural antisense RNA.

Furthermore, the results of the real-time PCR show that co-expression occurs in a cell type-specific pattern: the antisense/sense ratio is significant higher in NCF compared to NCM independent from culture time and passage of NCF. The fact of different antisense and sense expression

levels in various tissues has already been described for other genes, for example, for the basic Fibroblast Growth Factor (bFGF); in this case, it was shown that the natural antisense expression is tissue-specific. In addition, comparison of neonatal and adult rat tissue revealed that the expression is regulated developmentally [17].

In the last years, the question of the endogenous function of natural antisense transcripts has been discussed controversially. Our results are not in compliance with the classical view of natural antisense regulation which is a direct inhibition of protein translation. This direct inverse correlation between antisense and protein expression was already shown for several genes [18]. Based on this model, one would expect high absolute amounts of antisense RNA in NCF that do not express cTnI protein. Accordingly, the antisense RNA expression in NCM should be low. However, the northern blot analysis and real-time PCR showed high absolute amounts of both sense and antisense RNA in NCM. Additionally, western blot analysis revealed the expected cTnI protein signal in NCM, whereas no cTnI signal was detectable in NCF.

One possible explanation could be that not the absolute amount of antisense RNA but the relation between antisense and sense RNA (antisense/sense ratio) in one cell type defines whether the protein is translated or not. Indeed, the relative antisense RNA expression was higher in NCF independent from culture time and passage (Fig. 5(2)). Therefore, it is conceivable that natural antisense RNA has an inhibiting effect on cTnI translation in these cells, but this has to be proven in further experiments.

With regard to these results, a newer review by Lapidot and Pilpel is of special importance [19]. They summarize that the observation of negative and positive correlations of sense and antisense levels suggests that their mechanisms of action might be diverse. Furthermore, they describe four main models of natural antisense transcripts regulation: Transcriptional interference, RNA masking, chromatin remodeling, and finally double-stranded RNA-dependent mechanisms, which is the favored mechanism in our case. Moreover, they hypothesize that the relationship between natural antisense transcripts and their targets could indicate the regulatory mechanism that is in action [19].

As described before, we previously succeeded in the detection of antisense/sense-duplexes [7]. It is known that cells contain a specific enzyme apparatus acting on dsRNA [20]. One could assume that the antisense and sense RNA duplex formation in vivo induces a cascade of specific dsRNA-dependent nucleases which subsequently degrade the dsRNA. This could be an explanation why antisense and sense RNA amounts are relatively very low in NCF.

Previous analysis revealed that inhibition of cTnI protein translation takes place in vivo when cardiomyocytes are treated with ASO [7]. It is tempting to suggest that natural antisense RNA has the same effect, but it is likely that the regulation by natural antisense transcripts is much more complex.

An important finding of this study is the detection of natural antisense and sense RNA transcripts of the heart-muscle-specific protein cTnI in non-muscle cells like NCF. Two different methods, northern blot and RT-PCR, revealed the existence of cTnI-specific RNA in these cells. On the other hand, we could clearly reproduce the common fact that NCF do not express cTnI protein. The fact that natural antisense RNA is expressed in a cell type that does not express the gene at protein level is not unique: Robb et al. could detect a natural antisense transcript of the eNOS in vascular smooth muscle cells (VSMC) that normally do not express eNOS. In this regard, the activity of both the sense and antisense gene was shown in endothelial cells as well as in VSMC. Interestingly, eNOS protein expression was inducible in VSMC by a deactivation of natural antisense RNA with synthetic siRNA [13]. This result supports our hypothesis that antisense and sense transcripts of one gene are co-expressed in different cell types and that the antisense/sense ratio determines whether the gene is expressed at the protein level or not.

Nevertheless cell culture has to be discussed critically in this issue. In our previous studies, either whole heart tissue or isolated cardiomyocytes have been used for analysis. Here we tried to differentiate between muscle and non-muscle cells by applying a special protocol as described. The purpose of the culture was to achieve the highest possible purity of NCF. Usually, this is realized by several passage steps over at least 14 days. Since it is known that culture condition and duration can influence the expression of certain genes, we established a short-term culture protocol in order to reduce culture duration. We finally used NCF from passage 3 (short-term, 5 days of culture) for expression analysis. It is known that at passage 3 an enrichment of >98% of cardiac fibroblast is achieved [21]. To verify the purity of cell culture, we applied a Phalloidin assay that showed no obvious contamination by NCM. Therefore, it is unlikely that signals in RT-PCR and in the northern blot are caused by a contamination of cardiomyocytes. Furthermore, there are several other arguments that NCF do express antisense and sense RNA of cTnI. First, the antisense/sense ratio differs significantly between NCF and NCM. NCF showed a higher antisense/sense ratio independent from passage and culture time (Fig. 5(2)). Second, we performed western blot analysis and we could not detect cTnI protein, although antisense and sense RNA are expressed in these cells at very low levels.

Another important finding of the present study is the fact that besides the co-expression there is a tremendous excess of sense RNA in NCM and also in NCF. A 66-fold higher expression of sense RNA in NCM and a 12-fold higher

expression of sense RNA in NCF were shown in comparison to the related antisense RNA expression. This result points out to a co-regulation of sense and antisense RNA. One could hypothesize that there is a baseline expression of antisense and sense RNA and only if the sense expression exceeds a certain threshold the protein expression is enabled like in NCM. So natural antisense RNA could serve as a biological noise dampening element as hypothesized by some authors [19]. This could also explain the excess of sense RNA in NCF at lower levels (12-fold). In this case, the threshold for protein expression is obviously missed.

In this respect, the recent finding of co-regulation of antisense and sense RNA in MyHC is of importance [4]. Here a simultaneous increase of antisense and sense RNA of α-MyHC under T3 stimulation and, respectively, of β-MyHC under phenylephrine stimulation was shown. A coordinated regulation of antisense and sense RNA was already described for n-myc [22]. This is in accordance to our present results and supports the hypothesis that antisense and sense RNA of cTnI are not only co-expressed but also co-regulated in one cell type.

In summary, we could elucidate the expression pattern of natural antisense and sense RNA of cTnI in NCM and in NCF from different passages.

Our results revealed that antisense and sense RNA are co-expressed in both cell types. We could show that both RNA types are expressed simultaneously suggesting that the transcription of sense and antisense RNA is co-regulated. Comparing the antisense/sense ratios, we showed that the co-expression occurs in a cell type-specific pattern. The relative expression of antisense RNA was higher in NCF compared to NCM independent from culture time and passage. However, the absolute amounts of sense and antisense RNA were higher in NCM.

In conclusion, the cell type-specific co-expression of antisense and sense RNA of cTnI in NCM and NCF supports the hypothesis of a translation-modulating function of natural occurring antisense RNA.

Materials and methods

Cell preparation and cell culture

Heart tissue was taken from 2 to 3 days old Wistar rats. After excision, the tissue was digested with 50 μg/ml Trypsin (Gibco) in 10 ml calcium and magnesium-free Hank's Balanced Salt Solution (Gibco) for 18–20 h at 4°C. After stopping the reaction with 1 ml trypsin-inhibitor at 37°C, the tissue was dispersed with Worthington collagenase type 2 (Cell systems) for 45 min at 37°C. Cells were then washed with Leibovitz L-15 medium. After centrifugation, the pellet was resuspended in Medium 199 (Gibco) with 10% New Born Calf Serum (NBCS), 0.01 mM Ara-C and 100U/100 μg penicillin-streptomycin. The separation of NCM and NCF was achieved by preplating the cell suspension for 2 h at 37°C. The attached cells which are mainly NCF were used for fibroblast culture. NCM are enriched in the supernatant.

Cell culture of NCF

To investigate the influence of culture time of the fibroblasts, three different passages were analysed: NCF at passage 1 (2 days of culture), NCF at passage 3 (short-term culture, 5 days of culture), and NCF at passage 4 (long-term culture, 14 days of culture) since it is known that duration of cell cultivation can influence gene expression. The aim of the short-term culture was to achieve a high purity of NCF without contaminations of NCM, but with the shortest possible culture time. This was realized by a modified passage protocol.

At first, the attached NCFs were cultured in DMEM with 10% FCS (Gibco), 1% penicillin/streptomycin for 24 h at 37°C. After removing the medium and a washing step in PBS cells were detached by incubating with 2 ml trypsin/EDTA for 2 min at 37°C. Detached cells were then resuspended in 10 ml DMEM with 10% FCS. After centrifugation, the supernatant was again removed and cells were cultured as monolayers at a density of 1×10^6 cells/well on a six-well plate for 12 h. Afterward, these cells were cultured either according to the short-term or according to the long-term culture protocol. For short-term cultivation, NCFs were again detached with 1 ml trypsin/EDTA, resuspended and preplated for 2 h. Supernatant was again removed and cells were cultured for 48 h at 37°C. The same procedure was then repeated and cells were used as short-term cultured NCF at passage 3 after a cultivation of 5 days. Long-term cultured NCF were used at passage 4 after a cultivation of 14 days. These cells were passaged when necessary.

Cell culture of NCM

The NCM which are enriched in the supernatant were cultured in M199 with 10% FCS. The medium was enriched with cytosin-β-D-arabinofuranosid (Sigma) in order to reduce the number of divisible cells like fibroblasts in the culture. Cells were cultured as monolayers at a density of 1.5×10^6 cells/well on a six-well plate. Primary cultures of NCM were used for the experiments after a cultivation of 5 days.

Phalloidin assay

In order to ensure the purity of the NCF- and NCM-culture, a Phalloidin assay was performed. The cells were fixed

with 4% paraformaldehyd (Merck) and then made permeable by adding 1% Triton-X100. Afterward, the cells were stained with Phalloidin-FITC (Sigma) and with DAPI (4,6-diamidino-2-phenylindol) for 1 h at 37°C.

RNA isolation

The isolation of total RNA was performed using Trizol (Invitrogen) following the manufacturers protocol. Afterward, total RNA was treated with DNase I (Ambion) at 37°C for 30 min. Quality and quantity were checked using gel electrophoresis. In addition, ratio of optical density at 260 and 280 nm was >1.8 in all cases.

Specific RT-PCR

In order to distinguish between the sense and the antisense transcripts, we established a strand-specific RT-PCR. For the generation of cDNA, either sense-specific (R731) or antisense-specific (F20) primers were used (Fig. 7). The RT was performed on DNase I-treated total RNA (500 ng) with thermoscript reverse transciptase (Invitrogen) at a temperature of 50°C for 1 h in a volume of 20 µl. In order to check the efficacy of the RT-PCR reaction and for quantification of the DNA used, a reverse primer of the housekeeping gene GAPDH was added to the RT-reaction. Afterward, the generated sense- or antisense-specific cDNA was amplified with two different cTnI-specific primer pairs (F255/R731 and F255/R559) (Table 1) using a standard PCR-protocol with Nova Taq Hot Start DNA-polymerase (Novagen). PCR conditions were optimized for each primer pair. (F255/R731: annealing temperature: 66°C, 30 cycles; F255/R559: annealing temperature: 63°C, 35 cycles). For quantification, a second PCR with F/R-GAPDH was performed (annealing temperature: 59°C, 35 cycles).

Northern blot analysis

Up to 20 µg, total RNA was denaturated at 65°C for 30 min and then loaded on a denaturating 1% agarose gel (1× MOPS, 2% formaldehyde). After electrophoresis, the RNA was transferred onto a positively charged nylon membrane in 20× SSC for 18 h and crosslinked (254 nm, 120 mJ/cm^2). The membrane was pre-hybridised in DIG-EasyHyb (Roche) for 30 min at 68°C. For non-radioactive detection of the blotted RNA DIG-labeled cTnI RNA subcloned in two vectors pSPT18 and pSPT19 (Roche) was

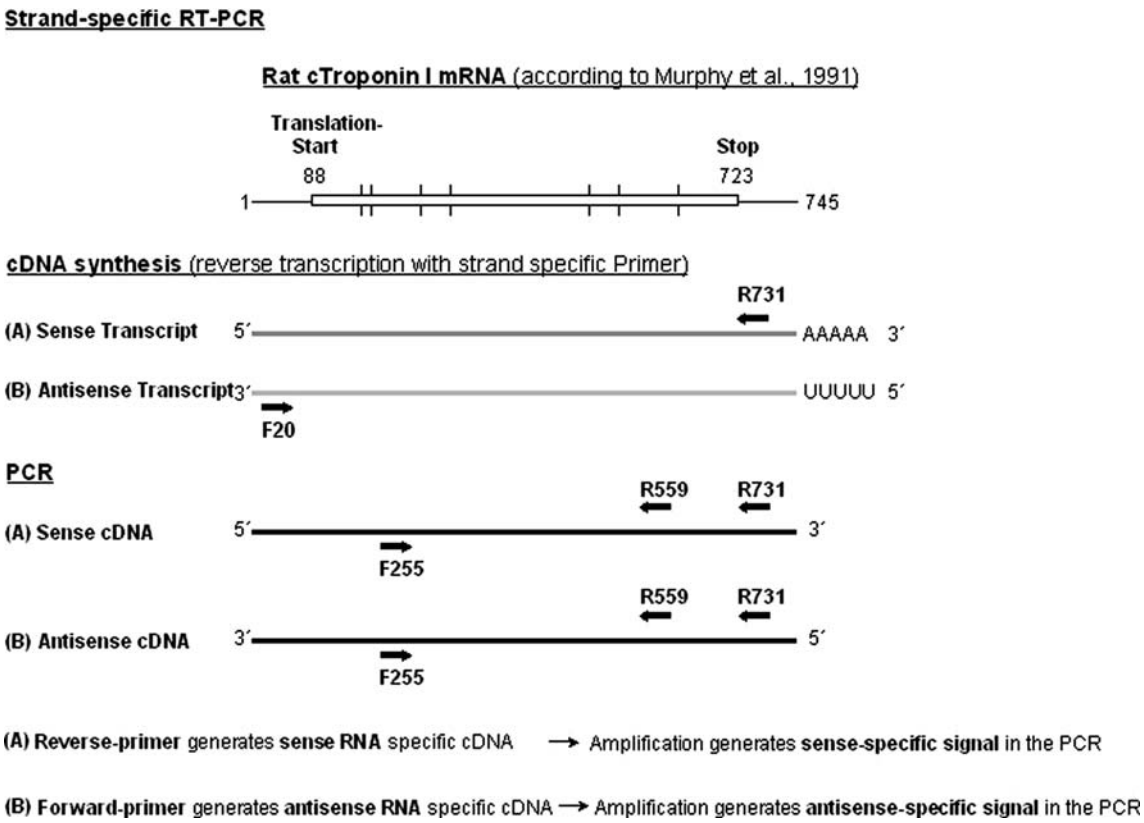

Fig. 7 Scheme of the strand-specific RT-PCR. Schematic figure of rat cardiac TnI mRNA sequence with coding exon sectors (open box) and spliced introns (vertical lines). The used antisense-specific (F20) and sense-specific (R731) cTnI primers for reverse transcription and cTnI primer pairs for cDNA amplification (F255/R731; F255/R559) are shown

transcribed in vitro with T7-polymerase (Roche) and DIG-UTP from the plasmids pSPT18-cTnI (for sense RNA) and pSPT19-cTnI (for antisense RNA). To obtain a positive control synthetic antisense and sense RNA were also produced by in vitro transcription using the same plasmids but normal UTPs instead of DIG-UTPs. Hybridisation was carried out overnight in DIG-Easy-Hyb (Roche) with 100 ng/µl of DIG-labeled RNA at 68°C for the sense-probe and at 62°C for antisense-probe. The RNA was detected by a chemiluminescense reaction with CSPD (Roche). In order to use the blot for another detection, a stripping procedure was carried out for 2 h at 80°C in a stripping solution (50% formamide, 25% SDS, 50 mM Tris/HCl, pH = 7.5) followed by a washing step in 2× SSC for 10 min at room temperature.

Real-time PCR

For real-time PCR the GeneAmp 5700 system (P.E. Applied Biosystems) and SYBR Green I dye were used. For quantification, the C_t-value of the TnI-probe was normalized against the C_t-value of a housekeeping gene as an external control. Hypoxanthine-guanine-phosphoribosyl-transferase (HPRT) was used in this case since validation experiments had shown that this housekeeping gene is expressed at nearly the same levels for sense and antisense RNAs in this tissue. Troponin I sense- and antisense-specific cDNA were generated performing a reverse transcription as already described above using TnI-F311 for antisense-specific cDNA generation and TnI-R731 for sense-specific cDNA generation. For semi-quantitative analysis, the housekeeping gene hypoxanthine-guanine-phosphoribosyltransferase (HPRT) was used. Similar amplification efficacy of the TnI-primer and HPRT-primer was checked by comparing the standard curves of both primer pairs. The real-time PCR was carried out in 25 µl volumes containing 2.5 µl SYBR GREEN PCR master mix (10×), 0.5 µl 10 mmol dNTPs, 200 nmol TnI-F509, 200 nmol TnI-R559 and 2.5 µl cDNA from RT-PCR. For amplification of the housekeeping gene, 200 nmol F-HPRT and 200 nmol R-HPRT were used. The cycle profile was 95°C for 10 min and then 40 cycles of 95°C for 15 s, 65°C for 1 min. All reactions were carried out in duplicate. Specificity of each amplified product was checked by analysis of the melting curve. Melting curves were generated automatically for every product by plotting the fluorescence signal as a function of temperature. Temperature peaks were 79°C for TnI-products and 81°C for HPRT-products, respectively.

Relative expression of antisense and sense RNA was calculated by ascertaining the delta C_t-value to HPRT and then using the formula $2^{(-\Delta C_t)} \times 100$.

Statistical analysis is based on $n = 3$ experiments (preparation of primary cell cultures of NCM and NCF). RNA was isolated from three different cell culture plates for each cell line and then used for reverse transcription and real-time PCR. Values are expressed as means ± SEM. Statistical significance was determined by one-way ANOVA including Bonferroni Post hoc Test for selected pairs. In the case of only two experimental groups, statistical significance was determined using the Student's t-test for unpaired groups. Differences in means with P-values <0.05 were considered statistically significant.

Western blot

About 10 or 20 µg isolated protein of NCM and NCF at passage 4 were separated by SDS-PAGE and then blotted on a PVDF-membrane by semi-dry blot. For specific detection of cardiac Troponin I, a polyclonal cTnI antibody (MA 1040/18-7,) 1:7000 as a primary antibody and peroxidase conjugated anti-mouse antibody 1:10000 as secondary antibody were used. The protein was detected by chemiluminescence reaction using the ECL-Kit (Amersham).

References

1. Yelin R, Dahary D, Sorek R, Levanon EY, Goldstein O, Shoshan A, Diber A, Biton S, Tamir Y, Khosravi R, Nemzer S, Pinner E, Walach S, Bernstein J, Savitsky K, Rotman G (2003) Widespread occurrence of antisense transcription in the human genome. Nat Biotechnol 21:379–386. doi:10.1038/nbt808
2. Luther HP, Morwinski R, Wallukat G, Haase H, Morano I (1997) Expression of sense and naturally occurring antisense mrna of myosin heavy chain in rat heart tissue and cultivated cardiomyocytes. J Mol Cell Cardiol 29:27–35. doi:10.1006/jmcc.1996.0248
3. Luther HP, Haase H, Hohaus A, Beckmann G, Reich J, Morano Ingo (1998) Characterization of naturally occurring myosin heavy chain antisense mRNA in rat heart. J Cell Biochem 70:110–120. doi:10.1002/(SICI)1097-4644(19980701)70:1<110::AID-JCB11>3.0.CO;2-T
4. Luther HP, Bartsch H, Morano I, Podlowski S, Baumann G (2005) Regulation of naturally occurring antisense RNA of myosin heavy chain (MyHC) in neonatal cardiomyocytes. J Cell Biochem 94:848–855. doi:10.1002/jcb.20319
5. Tobacman LS (1996) Thin filament-mediated regulation of cardiac contraction. Annu Rev Physiol 58:447–481. doi:10.1146/annurev.ph.58.030196.002311
6. Solaro R, Rarick HM (1998) Troponin and tropomyosin: proteins that switch on and tune in the activity of cardiac myofilaments. Circ Res 83:471–480
7. Podlowski S, Bramlage P, Baumann G, Morano I, Luther HP (2002) Cardiac troponin I sense-antisense RNA duplexes in the myocardium. J Cell Biochem 85:198–207. doi:10.1002/jcb.10116
8. Bartsch H, Voigtsberger S, Baumann G, Morano I, Luther HP (2004) Detection of a novel sense-antisense RNA-hybrid structure by RACE experiments on endogenous troponin I antisense RNA. RNA 10:1215–1224. doi:10.1261/rna.5261204
9. Rosok O, Sioud M (2004) Systematic identification of sense-antisense transcripts in mammalian cells. Nat Biotechnol 22:104–108. doi:10.1038/nbt925

10. Pittenger M (1994) Functional properties of non-muscle tropomyosin isoforms. Curr Opin Cell Biol 6:96. doi:10.1016/0955-0674(94)90122-8
11. Moses MA, Wiederschain D, Wu I, Fernandez CA, Ghazizadeh V, Lane WS, Flynn E, Sytkowski A, Tao T, Langer R (1999) Troponin I is present in human cartilage and inhibits angiogenesis. Proc Natl Acad Sci USA 96:2645–2650. doi:10.1073/pnas.96.6.2645
12. Manabe I, Shindo T, Nagai R (2002) Gene expression in fibroblasts and fibrosis. Involv Card Hypertrophy Circ Res 91:1103–1113
13. Robb GB, Carson AR, Tai SC, Fish JE, Singh S, Yamada T, Scherer SW, Nakabayashi K, Marsden PA (2004) Post-transcriptional regulation of endothelial nitric-oxide synthase by an overlapping antisense mRNA transcript. J Biol Chem 279:37982–37996. doi:10.1074/jbc.M400271200
14. Spencer CA, Gietz RD, Hodgetts RB (1986) Overlapping transcription units in the dopa decarboxylase region of Drosophila. Nature 322:279–281. doi:10.1038/322279a0
15. Shi M, Yan X, Ryan DH, Harris RB (2000) Identification of urocortin mRNA antisense transcripts in rat tissue. Brain Res Bull 53:317–324. doi:10.1016/S0361-9230(00)00349-X
16. Chen JJ, Sun M, Hurst LD, Carmichael GG, Rowley JD (2005) Genome-wide analysis of coordinate expression and evolution of human cis-encoded sense-antisense transcripts. Trends Genet 21:326–329. doi:10.1016/j.tig.2005.04.006
17. Li AW, Seyoum G, Shiu RPC, Murphy PR (1996) Expression of the rat BFGF antisense RNA transcript is tissue-specific and developmentally regulated. Mol Cell Endocrinol 118:113–123. doi:10.1016/0303-7207(96)03772-0
18. Kimelman D, Kirschner MW (1989) An antisense mRNA directs the covalent modification of the transcript encoding fibroblast growth factor in Xenopus oocytes. Cell 59:687–696. doi:10.1016/0092-8674(89)90015-9
19. Lapidot M, Yitzhak Pilpel (2006) Genome-wide natural antisense transcription: coupling its regulation to its different regulatory mechanisms. EuropMol Biol Org 7:116–1222
20. Kumar M, Carmichael GG (1998) Antisense RNA: function and fate of duplex RNA in cells of higher eukaryotes. Microbiol Mol Biol Rev 62:1415–1434
21. Turner NA, Porter KE, Smith WHT, White HL, Ball SG, Balmforth AJ (2003) Chronic β2-adrenergic receptor stimulation increases proliferation of human cardiac fibroblasts via an autocrine mechanism. Cardiovasc Res 57:784–792. doi:10.1016/S0008-6363(02)00729-0
22. Armstrong B, Krystal G (1992) Isolation and characterization of complementary DNA for N-cym, a gene encoded by the DNA strand opposite to N-myc. Cell Growth Differ 3:385–390