miR-193 expression differentiates telocytes from other stromal cells

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Abstract

Telocytes (TCs) are a particular type of interstitial (stromal) cells defined by very long, moniliform telopodes. Their tissue location, between blood vessels and other cells such as cardiomyocytes (CMC) and neurons, suggests a role in intercellular signalling. In order to define a microRNA (miR) signature in cardiac TCs, we have found that miR-193 is differentially expressed between TCs and other interstitial cells. Because miR-193 regulates c-kit, our data support the previous finding that TCs express c-kit in certain circumstances. In addition, the miRs which are specific to CMC and other muscle cells (e.g. miR-133a, miR-208a) are absent in TCs. Overall the data reinforce the view that TCs are a particular type of interstitial (mesenchymal) cells.

Keywords: telocytes • telopodes • microRNA • laser capture microdissection

Introduction

Telocytes (TCs) are a recently described type of stromal cells defined by their ultrastructural and morphological features [1]. Telopodes (particularly long and thin prolongations) are the hallmark of this cell type as seen in several organs by electron microscopy [2–8], and have also offered a strong indication of TCs’ presence in cell cultures of different origins [9–11]. Although it has been shown that various proportions of TCs express c-kit [12], CD34, caveolin-1 or VEGF [5] in vivo and in vitro, the identification of additional molecular markers is still a challenge. Therefore, we took advantage of laser capture microdissection (LCM), which offers the possibility of isolating a single cell with particular morphology from tissues or cultures and enables their further accurate investigation.

MicroRNAs (miRs) are small, single-strand RNA molecules that regulate protein levels by repressing gene translation [reviewed in 13]. miRs can show tissue- and cell-type-specific restriction [14], have distinct expression patterns during differentiation and are involved in various pathological processes. miR-1, miR-133a and miR-208a are preferentially expressed in cardiomyocytes (CMC) and other muscle cells and are necessary for proper skeletal and cardiac muscle development and function. They also play a role in cardiac hypertrophy, preconditioning or ischemia-reperfusion injury [15]. miR-199a is up-regulated in hypertrophic heart, when it is predominantly expressed in CMC, but was also detected at low levels in cardiac fibroblasts [16].

Several reports indicate that miR-21, miR-22, miR-29 and miR-193 are expressed in embryonic and adult fibroblasts [17, 18]. miR-21 is also detected in cardiac fibroblasts, with levels selectively increased in interstitial fibrosis and cardiac hypertrophy [19]. It has been shown that miR-29 plays a role in cardiac fibrosis through down-regulation of miRs encoding collagens and extracellular matrix proteins [20]. Very recently, miR-193 was found to repress c-kit expression in acute myeloid leukaemia, functioning as a tumour suppressor gene [21].

In this study, we have investigated whether miRs known to be present and to play a functional role in myocardium are expressed in TCs isolated by LCM from cultured murine cardiac cells.

Material and methods

Cell cultures

Healthy adult C57 black mice were used in accordance with ethical guidelines to harvest heart tissue. After mechanical mincing, small fragments...
were rinsed in HBSS without calcium or magnesium and incubated in type II collagenase (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 250 U/ml for 15 min. at 37°C. Collagenase was inhibited using ice-cold HBSS and the resulting cell suspension was centrifuged, washed in culture medium and seeded at a density of 1 × 10^5 cells/cm^2 in plastic culture dishes. Culture medium was DMEM-F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal calf serum. After 5 to 7 days, cells were detached using trypsin-ethylenediaminetetraacetic acid and replated at lower densities (1–2 × 10^5 cells/cm^2) in DuplexDish 50 plates (Carl Zeiss Microlmaging GmbH, Jena, Germany), a PEN membrane-covered culture dish that allows further laser microdissection of target cells.

We also used controls consisting of both 3T3 fibroblasts in culture and cell suspensions enriched in murine CMC obtained through gradient centrifugation of collagenase-dissociated murine heart tissue.

Laser capture microdissection

Cell cultures were washed with phosphate-buffered saline and then fixed with absolute ethanol, Giemsa stained and air-dried. This allowed for a better visualization of morphology details as compared to phase-contrast examination. LCM was performed with the PALM MicroBeam system (Carl Zeiss Microlmaging GmbH), with 10× to 40× objectives and in accordance with the manufacturer protocols. The criteria used for TC identification were: mononucleated small cells with at least one process longer than 50 μm and thinner than 0.5 μm, with moniliform dilations. Control cells with fibroblast morphology, but no telopodes, were also microdissected. In two separate experiments we harvested a total of 500 TCs and 500 control cells. Cells were catapulted into 500 μm tube caps filled with 30 μm of lysing and extraction buffer.

RNA extraction, reverse-transcription and microRNA qPCR

Total RNA was extracted with TRI Reagent (Sigma-Aldrich) and isolated using the protocol recommended by the manufacturer. The RNA samples were evaluated with NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

For first-strand cDNA synthesis we used QuantiMir RT (System Biosciences, San Francisco, CA, USA) according to manufacturer instructions. Basically, small RNAs were poly-A tailed, and an oligo-dT adapter was annealed to serve as a reverse-transcription primer. cDNA templates were then quantified in a real-time SYBR Green qPCR reaction (iCycler system and software, Bio-Rad, Hercules, CA, USA) using universal reverse primers and miR-specific forward primers. Mouse U6 snRNA and RNU43 were used as endogenous control assays and for data normalization. Threshold cycles were calculated for analysed miRs and subtracted from those of endogenous controls. Numbers obtained were subtracted from an arbitrary value, and the resulting score was used to assess specific miR relative expression levels.

Statistics

The data are represented as mean ± S.D. of three experiments and two-tailed P-values of less than 0.05 were considered as statistically significant.

Results and discussion

TCs are present in primary and first-passage cultures of murine cardiac tissue, as revealed by their typical morphology. Primary cultures had higher levels of debris and non-stromal cell types and reached 80% confluence 5 to 7 days after seeding. Two to 4 days after replating, variable numbers of TCs were visible, together with cells having ‘fibroblast-like’ morphology (e.g. spindle shaped, with a few short and thick end-processes).

Giemsa-stained TCs grown on membranes can be easily identified and accurately microdissected. Figure 1 shows the typical morphology of TCs that were harvested, as well as cells that were used as a negative control for miR expression relative quantification. Cells were isolated within 48 hrs of their staining and kept at 4°C between procedures.

In order to validate our method in terms of specificity and sensitivity, we have tested the expression of several miRs known to be preferentially expressed in different types of cells within cardiac tissue (Fig. 2). Thus, the CMC-specific miRs, miR-1-133a and miR-208a, show high level of expression in the samples enriched in CMC (Fig. 2A), whereas miR-21, miR-29 and miR-199a-5p are preferentially expressed in cultured cardiac cells (CCC) and in fibroblasts (Fig. 2B). Noteworthy, miR-193 is expressed at lower level in CCC than in 3T3 fibroblasts, suggesting that, besides of cardiac fibroblasts, the cultures may contain cells where this miR is down-regulated (Fig. 2B).

CMC-specific miRs are not expressed by TCs, as expected. Microdissected TCs and control cells did not express any of the CMC-specific miRs (miR-1, 133a or 208a), but, in accordance with their mesenchymal origin, showed various levels of miR-21, 22, 29 and 199a-5p (Fig. 3A).

TCs do not express miR-193. In all experiments, miR-193 expression was below the detection level in microdissected TCs, whereas neighbouring fibroblasts-like cells were positive for it (Fig. 3B). The result correlates with the lower detection of miR-193 in CCC than in fibroblasts (Fig. 2B) because the cardiac culture contains both types of cells, positive and negative for miR-193. The data support the view that TCs are a distinct cell type also in terms of gene expression and regulation pathways. Noteworthy, c-kit is predicted by TargetScan (http://www.targetscan.org) to be a target of miR-193, an interaction that has been validated experimentally [21]. Thus, our results concur with previous observations that cardiac TCs can be differentiated in situ from surrounding stromal cells by their c-kit expression [12]. Other predicted targets of miR-193 include matrix metallopeptidase 19-an enzyme that facilitates cell motility by breaking down extracellular matrix in normal processes such as tissue remodelling and in pathological circumstances [22]; p guanine nucleotide exchange factor 12-a GTPase involved in repulsive guidance of cell migration [23] and the chromobox protein CBX7 that positively regulates E-cadherin expression by interacting with the histone deacetylase protein 2 [24].
In conclusion, a functional picture of TCs is emerging, where this particular type of stromal cells is responsible, as opposed to canonical fibroblasts, for establishing and maintaining tissue scaffold that is involved in cell guidance and cell-to-cell signalling. Moreover, we think that TCs act in tandem with cardiac (progenitor) stem cells [25, 26].

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