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TERT over-expression affects the growth of myocardial tissue derived from mouse embryonic stem cells

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ABSTRACT

For heart failure, new therapeutic strategies based on augmentation of the regenerative resources of the heart muscle are under evaluation. More knowledge about the mechanisms regulating growth of the embryonic or adult heart muscle will help to improve the results. The present over-expression study provides further insight into the role of telomerase reverse transcriptase (TERT) in growth regulation of myocardial tissue derived from embryonic stem (ES) cells.

Mouse ES cells (D3) exhibiting ectopic expression of TERT under the regulation of the β-actin promoter were generated and allowed to differentiate over a period of up to 18 days. In contrast to the controls, the TRAP assay did not reveal any decrease of telomerase activity during differentiation of TERT transgenic ES cells. Following cell dissociation and staining for sarcomeric α-actinin, singular myocardial precursors could be identified and analyzed using fluorescence microscopy: compared with the controls, the outgrowths of TERT transgenic ES cells showed a significant enlargement of the cellular fraction formed by cardiomyocyte precursors, while BrdU-(double) staining did not reveal a change of its proliferation rate. In addition, the average physical dimensions of the precursors appeared to be enlarged. The myocardial precursors exhibited three different morphologies: spindle-like or round or tri-/multi-angular. While, compared with the controls, in TERT transgenic ES cell outgrowths the overall number of myocardial cells was enhanced, the formation of spindle-like or round-shaped precursors was suppressed. On the molecular level, RT-PCR analysis showed the mRNA-expression level of α-MHC, a gene whose expression is specific for pacemaker-like or atrial-like precursors (Kolossov et al., 2005), to be reduced. Furthermore, TERT transgenic outgrowths displayed a reduced beating frequency.

It can be concluded that TERT over-expression promotes the differentiation of mouse ES cell-derived cardiomyocytes in a phenotype-specific manner.

1. Introduction

Ischemic heart disease is one of the leading causes of morbidity and mortality in the developed world. For pharmacologically intractable failing hearts, allogenic transplantation is the treatment of choice. However, the disparity between the number of donors and recipients limits this approach. There is evidence that even the adult human heart contains a population of cardial stem cells (CSC), which can divide and differentiate into cardiomyocytes and smooth muscle cells and endothelia cells (Beltrami et al., 2001; Quaini et al., 2004; Urbanek et al., 2005). In chronic ischemic heart disease, however, the CSC growth is likely to be impaired by cellular senescence and increased apoptosis, resulting in a decline of the number of functionally competent cells (Urbanek et al., 2005). This mechanism may underlie the progressive functional deterioration and the onset of terminal failure.

Nevertheless, the knowledge that the adult heart has the potential of regeneration via stem cells opens a new window for therapeutic strategies in heart failure, based on augmentation of the intrinsic repair mechanism. Thus, CSC actually present in the heart may be activated or supplemented by the differentiation of exogenic embryonic or adult stem cells, transplanted to the damaged heart tissue. As proposed by proof-of-concept studies, stem cells should be directed to some degree towards the cardiomyogenic lineage in vitro prior to transplantation, to improve the functional benefits to the heart or to reduce undesirable site effects (Heng et al., 2004). The isolation of cardiomyocyte precursors from other cell lineages prior to transplantation may be an even more promising approach (Klug et al., 1996; Min et al., 2002, Kolossov et al., 1998; Sachinidis et al., 2003; Gulbins et al., 2002; Zandstra et al., 2003; Bohler et al.,

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2005), which, however, is hampered by the difficulties to produce precursors in a large scale sufficient to repair a myocardial infarction in humans, by the fact that the engraftment of the precursors accounts for only a modest regeneration of myocardium in the infarcted heart (Min et al., 2002), and by the high rate of (immunological) rejection after transplantation (Odorico et al., 2001). More knowledge about the mechanisms regulating differentiation and growth of heart tissue could help to improve stem cell-based therapeutic strategies in heart failure.

In this regard, a couple of studies have focused on telomerase. Telomerase is an intra-nuclear ribonucleoprotein, which elongates the G-rich ends of telomeres by reverse transcription of an internal template RNA (Linger et al., 1997). Telomeres are specialized DNA–protein complexes, which cap the ends of eukaryotic chromosomes and are essential for maintaining the chromosomal integrity and therewith the viability of cells (Gall, 1991). Telomeres have been shown to shorten down, due to repeated cell division (Harley et al., 1990) or DNA damage (Von Zgelinicki et al., 1995), until, when a certain length is reached, cell cycle exit and /or apoptosis is triggered (Harley, 1991). If this so-called "Hayflick limit" is overridden, further progressive shortening of telomeres will lead to chromosomal instability and death of the cells (Crisis) (Counter et al., 1992). Activation of telomerase can prevent functional loss of telomeres (Counter et al., 1992), providing evidence for its ability to immortalize cells. The telomerase reverse transcriptase (TERT) is the only structural subcomponent of telomerase, whose expression is limited (Jun-Ping, 1999), implying its important role in regulation of telomerase activity. In addition, TERT it is thought to exert activities in the cells independent of the functional telomerase complex (Cao et al., 2002). Elongation of the replicative life span, enhancement of proliferation, protection from apoptosis, as well as alterations in differentiation potential are general effects that are attributed to telomerase activity and/or the presence of TERT in normal or progenitor cells (Mattson et al., 2001; Simonden et al., 2002; Smith et al., 2003). Furthermore, the TERT expression level has been shown to correlate with the mobilisation efficiency of adult stem cells (Flores et al., 2005).

TERT is highly expressed in stem cells, but is down-regulated during differentiation in most somatic tissues, accompanied by a decrease of telomerase activity (Armstrong et al., 2000). Counteracting the decrease of TERT expression in the developing mouse heart by ectopic (over)expression of TERT under the regulation the α-MHC promoter leads to an increase of the number or diameter of the cardiomyocytes. In addition, the prevalence of apoptosis after coronary ligation and the subsequent area of infarction is reduced (Oh et al., 2001). On the other hand, heart muscle cells of telomerase knockout mice in the second generation show an attenuation of proliferation and an increase of apoptosis (Leri et al., 2003). Furthermore, it has been hypothesized that postnatal telomere attrition may represent an early origin of later cardiovascular disease (Aviv, 2002). Compared with controls, TERT expression has been demonstrated to be unchanged in acute and reduced in chronic-infarcted human hearts, while telomerase activity has been shown to be elevated 8.6-fold in acute and 2.6-fold in chronic infarcts and to correlate positively with the mitotic indices of the CSC (Urbanek et al., 2005).

Taken together, the cited studies point out that telomerase activity and/or TERT expression are key factors in a highly complex system controlling primary and adaptive growth of heart tissue.

The present over-expression study provides evidence that TERT can alter the potential of (mouse) embryonic stem cells to differentiate into cardiomyocyte precursors in a subtype-specific manner.

2. Material and methods

2.1. Transfection construct

In order to realize an ectopic expression of telomerase reverse transcriptase (TERT) in mouse embryonic stem cells (ES), which is coupled with EGFP expression and driven by the β-actin promoter, an expression vector was constructed, containing a β-actin-promoter-mTERT-IRES-EGFP cassette (TERT+) (Fig. 1A).

pIRES2-EGFP (Clontech) was used as vector-backbone, containing a SV40-neomycin cassette for positive selection in G418. The CMV I promoter was excised, using Ase I and Nhe I. mTERT cDNA (3.5 kb), kindly provided by Robert A. Weinberg, Ph.D., Member of Whitehead Institute, Professor of Biology, Massachusetts Institute of Technology, MIT, Cambridge/USA, was introduced by EcoRI – Sal I digestion into the MCS of the promoter-less vector-backbone upstream of the IRES-region. In a second clonig step, the β-actin promoter was blunt-end inserted upstream of the mTERT-IRES-EGFP-cassette, following an other digestion with EcoRI. Transformation was carried out in *Escherichia coli*. Plasmid DNA was prepared using the Qiagen Midiprep Kit and linearized using SacI. The same procedure was followed for transfection of the construct with the TERT cDNA-insert left out, used as control (TERT−) (Fig. 1A).

2.2. ES cell culture and embryoid body formation

The mouse ES cell line (D3) (Doetschmann et al., 1985), used for this study, was kept undifferentiated on confluent feeder layers of mitomycin-treated primary culture of murine embryonic fibroblasts by addition of purified recombinant mouse leukaemia inhibitory factor (1000 units/ml medium). It was used Dulbecco's modified Eagle medium (DMEM), supplemented with 15% heat-inactivated fetal calf serum, 1% non-essential amino acids, 50 U/ml Penicillin/Streptomycin (all reagents from GIBCO BRL, Germany), and 0.1 mM β-mercaptoethanol (Sigma, Germany). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Monolayers were passaged by trypsinization.

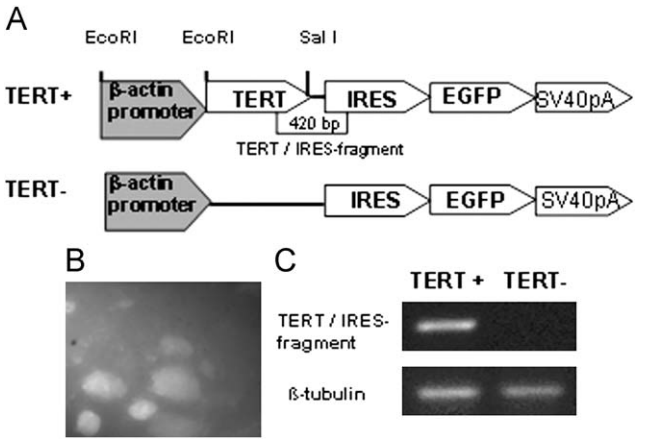

Fig. 1. (A) TERT transgenic D3 cells were generated by transfection of an expression-vector (TERT+) containing a cassette that comprises the β-actin promoter as well as the mTERT- and EGFP-gene. Both genes are linked by the IRES segment. Restriction sites used for insertion of the promoter and the mTERT cDNA are marked. Primers used for RT-PCR in order to test for ectopic TERT mRNA-expression in the transfected cells span a 420 bp fragment overlapping the IRES and TERT sequences. In the control vector (TERT−), the insertion of the mTERT-gene was left out. (B) Colonies of transfected D3 cells show green colouring under fluorescence illumination, due to the expression of EGFP. (C) Lane 1: IRES-TERT-mRNA can be detected using RT-PCR in transgenic (TERT+) D3 cells, but not in D3 cells, into which the control vector (TERT−) was transfected. lane 2: mRNA-level of the house-keeping gene β-tubulin.