Embryonic stem cell-derived cardiomyocytes
Fijnvandraat, A.C.

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From blastocyst stage embryos, germ cell tumors and primordial germ cells, embryonic pluripotent cell lines have been derived known as embryonic stem (ES) cells, embryonic carcinoma cells and embryonic germ cells, respectively. They can be cultured indefinitely in an undifferentiated state, a prerequisite for being a genuine stem cell. Upon differentiation in vitro, derivatives of all three germ layers (ectoderm, mesoderm and endoderm) are formed, including cardiomyocytes. For ES cells, withdrawal of differentiation inhibiting factors like LIF, and aggregation to form so-called embryoid bodies, are of eminent importance in the process of in vitro differentiation. In chapter 1 the different embryonic cell lines, culture methods and applications are discussed.

In vitro differentiation of ES cells can be applied as a model system to analyse genes and (growth) factors involved in early developmental processes, including cardiogenesis. In vitro differentiation of (human) ES cells is also mentioned as a method to acquire donor cells for cellular transplantation to treat multiple diseases like cardiac infarction. In this respect, several studies are noteworthy that report that even in the adult body stem cells are present which are able to differentiate into cardiomyocytes upon engraftment in the heart. Most known are the haematopoietic and bone marrow stem cells. However, ES cells can be cultured to large amounts in vitro and their differentiation potential is higher compared to that of adult stem cells, from which differentiation results are not beyond discussion as yet.

The use of ES cells as a tool in basic cardiac research and as a source of new cardiomyocytes for transplantation in the patient requires proper characterisation of these in vitro produced cardiomyocytes, because their developmental progress determines their options and limitations. Incompletely differentiated ES cells are prone to tumours and arrhythmias upon transplantation in the heart. Therefore, a central issue in this study is to assess the degree of differentiation and maturation of in vitro differentiated cardiomyocytes. To what extent differentiation in vitro relates to in vivo development is not known very well as yet, although several studies in the past investigated this issue. These studies compared gene expression and electrical currents between embryonic stem cell-derived cardiomyocytes and the in vivo formed heart. These comparative studies did not take into account molecular and electrophysiological characteristics of the developing heart. This, however, is important because ES cells are supposed to go through a developmental progress during differentiation. It is important to note that many genes that are specific for a cardiac compartment in adult life are not so in the embryo, and cannot be used as such without due consideration. Chapter 2 gives an overview of the morphological, functional and molecular development of the heart. This provides a basis to compare ES cell-derived cardiomyocytes and cardiomyocytes that develop in vivo.

A parameter to approach the degree of maturation is the level of gene expression. Chapter 3 describes a sensitive real time reverse transcription PCR method, which
Summary

enables the detection of low expression levels of genes in differentiating ES cells quantitatively. In homogenates of embryoid bodies, however, spatial distribution of gene expression is lost. Non-radioactive in situ hybridisation coupled to a tyramide-mediated amplification step allowed visualisation of the low abundant gene expression patterns as observed in embryoid bodies, even at the level of single cells, as described in chapter 4. In this way, the degree of heterogeneity of ES cell-derived cardiomyocytes could be assessed.

The above-mentioned methods were applied in chapter 5 and 6. It was found that about 30% of the cells of an embryoid body are cardiomyocytes. Based on the co-localisation and the levels of expression of distinct cardiac genes and transcription factors in embryoid bodies and in embryonic mouse hearts, it was concluded that ES cell-derived cardiomyocytes are most reminiscent of cells of the embryonic heart tube, at the stage that the process of chamber formation has just been initiated.

In addition to the molecular approaches for phenotyping the ES cell-derived cardiomyocytes, we used patch clamp and the voltage-dependent fluorescent dye ANEPPS to characterise ES cell-derived cardiomyocytes electrically. Many studies have reported that ES cell-derived cardiomyocytes show atrial-like or ventricular-like characteristics. Whether these cells are like those of adult atrial or ventricular chamber myocardium, or like those of the embryonic myocardium of the heart tube remained unclear, because electrophysiological data of embryonic mouse cardiomyocytes were not known as yet. Therefore, we have assessed mouse action potential characteristics of distinct regions of embryonic day 12.5 hearts (outflow tract, atrium, ventricle). The action potential parameters of relatively young ES cell-derived cardiomyocytes were most similar to those of the embryonic outflow tract, which consists of myocardium, which is similar to that of the primitive embryonic heart tube. Later during differentiation, these cardiomyocytes were most reminiscent of cells of the embryonic atrium. No cells were detected similar to embryonic ventricular cells.

Upon differentiation in vitro, many phenotypes arise apart from cardiomyocytes. It is interesting to be able to study cardiomyocytes without other potentially influencing cell types. When ES cell-derived cardiomyocytes would be applied for transplantation, pure cultures are essential. A method has been developed to isolate a pure population of cardiomyocytes, as described in chapter 6. To this end an ES cell line was created, stably transfected with an antibiotic resistance gene under direction of a cardiac-specific promoter fragment. After a selection procedure an almost 100% cardiomyocyte population was established. Using these cells for electrophysiological characterisation, an initiation of the differentiation towards chamber myocardial cells was observed.

We have demonstrated that early stages of chamber myocardial cells develop in vitro, as discussed in chapter 5 and 6. An important transcription factor involved in the
formation of chamber myocardium is \textit{Tbx5}. In \textbf{chapter 7}, overexpression of this transcription factor in a model system of P19Cl6 embryonic carcinoma cells has been described. It became clear that variation between separate clones and differentiation cultures makes an \textit{in vitro} differentiation system elaborate. Nevertheless, it has been concluded that the chamber gene-inducing activity of \textit{Tbx5} could be recapitulated \textit{in vitro}. This might be a first step towards the development of \textit{in vitro}-produced chamber myocardial cells. Hope stems for broken hearts!\textsuperscript{1}

\textsuperscript{1} Helen Pearson (2001) Science, March 31\textsuperscript{st}