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REGULAR ARTICLE

Derivation of functional ventricular cardiomyocytes using endogenous promoter sequence from murine embryonic stem cells

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Abstract The purpose of this study is to establish a murine embryonic stem cell (mESC) line for isolation of functional ventricular cardiomyocytes (VCMs) and then to characterize the derived VCMs. By crossing the myosin light chain 2v (Mlc2v)-Cre mouse line with the reporter strain Rosa26-yellow fluorescent protein (YFP), we generated mESC lines from these double transgenic mice, in which Cre-mediated removal of a stop sequence results in the expression of YFP under the control of the ubiquitously active Rosa26 promoter specifically in the VCM. After induction of differentiation via embryoid body (EB) formation, contracting YFP^+ cells were detected within EBs and isolated by fluorescence-activated cell sorting. N-cadherin, the cadherin expressed in cardiomyocytes, and the major cardiac connexin (Cx) isoform, Cx43, were detected in the respective adherens and gap junctions in these VCMs. Using current clamp recordings we demonstrated that mESC-derived VCMs exhibited action potential characteristics comparable to those of neonatal mouse VCMs. Real-

Abbreviations: AP, action potential; APA, action potential amplitude; APD, action potential duration; CMV, cytomegalovirus; MDP, maximum diastolic potential; cTnT, cardiac troponin T; EB, embryoid body; ECC, embryonic carcinoma cell; ESC, embryonic stem cell; GFP, green fluorescent protein; MEF, mouse embryonic fibroblast; MHC, myosin heavy chain; MI, myocardial infarction; MLC2v, myosin light chain 2v; VCM, ventricular cardiomyocyte; YFP, yellow fluorescent protein.

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time intracellular calcium $[Ca^{2+}]_i$ imaging showed rhythmic intracellular calcium transients in these VCMs. The amplitude and frequency of calcium transients were increased by isoproterenol stimulation, suggesting the existence of functional β-adrenergic signaling. Moreover, $[Ca^{2+}]_i$ oscillations responded to increasing frequencies of external electrical stimulation, indicating that VCMs have functional excitation–contraction coupling, a key factor for the ultimate cardiac contractile performance. The present study makes possible the production of homogeneous and functional VCMs for basic research as well as for cardiac repair and regeneration.
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Introduction

Despite recent advances in pharmacological and surgical therapies, heart disease is the leading cause of mortality and morbidity worldwide (Jessup and Brozena, 2003). Cardiac-related deaths due to ischemic heart disease are caused by occlusion of a coronary artery resulting in ventricular myocardial infarction (MI), which induces extensive myocardial cell death within the ischemic zone (Freude et al., 2000; Scarabelli et al., 2001), leading to heart failure (Gepstein, 2010). Cell-based therapies are considered one of the most promising solutions to regenerate the damaged heart tissue. In the last decade, several groups reported that transplantation of isolated fetal or neonatal cardiomyocytes can aid in the regeneration of damaged heart tissue (Müller-Ehmsen et al., 2002; Roell et al., 2002). However, these studies were limited by the availability of fetal and neonatal cardiomyocytes (Reinecke et al., 2008). Current research seeks to address these issues by actively pursuing new strategies to derive novel/additional cell types useful for regenerative medicine from stem cells.

Embryonic stem cells (ESCs) are derived from the inner cell mass of the blastocyst stage embryos (Evans and Kaufman, 1981), and they have the ability to produce cells of all three germ layers, including heart cells (Wobus et al., 1997). Thus, they can provide an abundant, renewable source of cells for cardiac repair. Efforts have been made to develop strategies for cardiac lineage selection and cardiomyocyte differentiation using ESCs. Heterogenous cultures containing cardiac cells can be isolated from ESCs in culture (Kehat et al., 2001; Laflamme et al., 2007; Maltsev et al., 1994; Mummery et al., 2003; Xu et al., 2002). Commonly, during cardiac differentiation in ESCs, all cardiac phenotypes including nodal, atrial and ventricular cells can be detected in ESC cultures (Laflamme et al., 2007; Yang et al., 2008). However, this phenotypic heterogeneity of differentiated ESC samples might lead to an inefficient engraftment and might cause abnormal electrical activity after implantation (Zhu et al., 2010; Hansson et al., 2009). Thus, it is critically important to isolate highly purified cardiac cells.

Ventricular cardiomyocytes (VCMs) are the most extensively injured cardiac cell type in ischemic heart disease and, as a result, the leading cause of reduced cardiac function. Therefore, it is of great interest to generate a renewable source of VCMs from ESCs for cell-based therapies to treat heart failure. Myosin light chain 2, ventricular isoform (*Mlc2v*) is specifically expressed in the ventricular chamber and is required for ventricular chamber morphogenesis during mammalian cardiogenesis (Chen et al., 1998; Minamisawa et al., 1999), providing an ideal marker for the isolation of VCMs. By using an artificial reporter system in which enhanced green fluorescent protein (*eGfp*) is driven by a hybrid promoter composed of a fragment of *Mlc2v* promoter and the enhancer element of the cytomegalovirus (CMV), two groups have previously established transgenic murine ESC (mESC) and embryonic carcinoma cell (ECC) lines for the isolation of VCMs, respectively (Moore et al., 2004; Müller et al., 2000). In the present study, we established a stable reporter system using endogenous promoter specifically activated in VCMs.

The *Mlc2v-Cre* mouse line, in which *Cre* is knocked into one of the endogenous *Mlc2v* loci, is a well-established strain for marking VCMs (Chen et al., 1998; Minamisawa et al., 1999). By breeding this *Cre* line with the conditional *Cre* reporter strain *Rosa26*-yellow fluorescent protein (*Yfp)* (Abou-Khalil et al., 2009), we generated ESC lines from the blastocysts of the double transgenic embryos (*Mlc2v-Cre*; *Rosa26-Yfp*) and isolated VCMs from these ESC lines by using in vitro differentiation. In this study, Cre-mediated removal of a stop sequence resulted in the expression of YFP under the control of endogenous *Rosa26* promoter specifically in VCMs. We further showed that these ESC-derived VCMs displayed the capacity to form the functional syncytium, neonatal ventricular cardiomyocyte-like action potentials, and rhythmic intracellular calcium transients that are responsive to both chemical and electrical stimulation. This mESC line will allow the production of homogeneous, functional VCMs for cell-based ventricular repair and regeneration in murine heart injury models. This study will set the stage for the isolation of human VCMs using MLC2V as marker in human ESCs and induced pluripotent stem cells (iPSCs) for use in cardiac repair.

Results

Establishment of *Mlc2v-Cre*; *rosa26-Yfp* mESC line

We generated a mESC line using conditional genetic lineage tracing. *Mlc2v-Cre* mice were crossed into the conditional Cre reporter Rosa26-YFP strain. We isolated the *Mlc2v-Cre*; *Rosa26-Yfp* mESC line from day 3.5 embryos (Fig. 1A) and confirmed the presence of the *Mlc2v-Cre* and the *Yfp* genes in this mESC line by genomic DNA PCR analysis. Conversely, wild-type mice express neither *Mlc2v-Cre* nor the *Yfp* genes (Fig. 1B). Additionally, this *Mlc2v-Cre*; *Rosa26-Yfp* mESC line has a normal karyotype (Fig. 1C). To induce cardiac differentiation, embryoid bodies (EBs) derived from mESCs were cultured as previously described (Qyang et al., 2007). After 6 days following EB formation, scattered YFP expression was detectable in EBs. In order to examine whether YFP expression correlates with the presence of MLC2v proteins, mESC-derived beating cluster at day 10 was immunostained with

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