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Transformation of jaw muscle satellite cells to cardiomyocytes

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ABSTRACT

In the embryo a population of progenitor cells known as the second heart field forms not just parts of the heart but also the jaw muscles of the head. Here we show that it is possible to take skeletal muscle satellite cells from jaw muscles of the adult mouse and to direct their differentiation to become heart muscle cells (cardiomyocytes). This is done by exposing the cells to extracellular factors similar to those which heart progenitors would experience during normal embryonic development. By contrast, cardiac differentiation does not occur at all from satellite cells isolated from trunk and limb muscles, which originate from the somites of the embryo. The cardiomyocytes arising from jaw muscle satellite cells express a range of specific marker proteins, beat spontaneously, display long action potentials with appropriate responses to nifedipine, norepinephrine and carbachol, and show synchronized calcium transients. Our results show the existence of a persistent cardiac developmental competence in satellite cells of the adult jaw muscles, associated with their origin from the second heart field of the embryo, and suggest a possible method of obtaining cardiomyocytes from individual patients without the need for a heart biopsy.

1. Introduction

In embryonic development the heart forms from three separate regions of the early mesoderm. The primary heart field gives rise to the atria and left ventricle, while the second heart field gives rise to the right ventricle and outflow tract, and, at least in the chick embryo, there is also a third heart field forming the pacemaker region (Bressan et al., 2013; Buckingham et al., 2005). The second heart field is part of the cranial splanchnic mesoderm and, in addition to forming parts of the heart, also forms the jaw muscles of the head (Buckingham, 2001; Grifone and Kelly, 2007; Lescroart et al., 2010; Nathan et al., 2008). The second heart field is characterized by expression of the transcription factor Islet1 (ISL1) (Black, 2007; Cai et al., 2003; Laugwitz et al., 2008, 2005; Moretti et al., 2006).

In skeletal muscle, stem cells, called satellite cells, are the source of new muscle fibers during normal growth and are also responsible for generating new fibers following muscle damage in adult life (Dhawan and Rando, 2005; Hawke and Garry, 2001; Morgan and Partridge, 2003; Seale and Rudnicki, 2000). As might be expected from the different origins of the parent muscles, the jaw and trunk skeletal muscle satellite cells are also derived from distinct embryologic cell populations and may have different regenerative abilities (Gayraud-Morel et al., 2012). Trunk and limb muscle satellite cells originate from the somites (Daughters et al., 2011; Gros et al., 2005; Relaix et al., 2005), while satellite cells of the jaw muscles originate from the second heart field (Harel et al., 2009; Nathan et al., 2008; Sambasivan et al., 2009). Because of the close developmental relationship between jaw muscles and heart, we postulated that the satellite cells of the jaw muscles in adult animals might retain aspects of secondary heart field developmental competence and still be capable of developing into cardiomyocytes.

Here we show, for the first time, that it is possible to take satellite cells from jaw muscles of the adult head and to direct their differentiation to become embryo-type cardiomyocytes. This is done by exposing the cells to extracellular factors similar to those which heart progenitors would experience during normal embryonic development. By contrast, cardiac differentiation does not occur at all from satellite cells isolated from trunk and limb muscles, which originate from the somites of the embryo. The cardiomyocytes arising from jaw muscle satellite cells express a range of specific marker proteins, beat spontaneously, display long action potentials with appropriate responses to nifedipine, norepinephrine and carbachol, and show synchronized calcium transients. Our results show the existence of a persistent cardiac developmental competence in satellite cells of the adult jaw muscles, associated with their origin from the second heart field of the embryo, and suggest a possible method of obtaining cardiomyocytes from individual patients without the need for a heart biopsy.

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2. Materials and methods

2.1. Cell cultures

All animal procedures were conducted under IACUC protocol 1002A78295. Satellite cell-derived myoblasts of the jaw (digastric and masseter) or from trunk (limbs and back) muscles were isolated by magnetic cell sorting (MACS) or fluorescence activated cell sorting (FACS) using sequential sorting for CD45, CD31, SCA1 negative, followed by positive selection for integrin β-1 and integrin α-7 cells, as previously described (Hirai et al., 2010). Myoblasts were cultured in myoblast medium containing 20% fetal bovine serum (FBS) (Asakura et al., 2002) supplemented with 100 ng/ml bone morphogenetic protein 4 (BMP4) (R & D Systems) plus 10 ng/ml basic fibroblast growth factor (bFGF) (R & D Systems) for four days followed by 100 ng/ml dickkopf 1 (DKK1), 10 ng/ml bFGF and 5 ng/ml vascular endothelial growth factor (VEGF) for two days and allowed to differentiate in low serum Dulbecco's minimal essential medium (DMEM) (Gibco) with 5% FBS or serum free (StemPro, Gibco) to a total of twenty-one days. Some initial experiments were conducted using a modified differentiation scheme that consisted of myoblast medium supplemented with 100 ng/ml BMP4 for two days followed by 100 ng/ml DKK1 for three days.

Movies of beating cardiomyocytes were taken from day 21 cultures isolated from the myotubes and re-plated in differentiation medium for 2 days. Video was recorded at 7 frames/sec for 180 s. For single-fiber muscle preparations, masseter or digastric muscles were removed and incubated in 0.2% type I collagenase in DMEM for 45 min at 37 °C. Isolation of single fibers was achieved through mechanical disruption. They were cultured in myoblast medium for 12 h with or withour BMP4 (100 ng/ml) and then immunostained with antibodies for PAX7 and NKX2.5.

2.2. RNA and gene expression

Two step RT-PCR was performed on an ABI Prism 7500 Real Time PCR System (Applied Biosystems, Foster City, Calif.). Total RNA was isolated from cultured cells during the differentiation protocol using Trizol reagent (Invitrogen). cDNA was generated from 5 μg of total RNA using 1st Strand Synthesis Supermix primed with random hexamers (Invitrogen). Relative qRT-PCR was performed on 1 μl of cDNA using qRT-PCR SYBER Green Master Mix (Invitrogen) with murine specific primers for myogenic (*Pax7, M-Cad, Myf5, MyoD*), early cardiac progenitor cell (*Nkx2.5, Tbx5, Gata4, Isl1, Tbx1*), or late cardiomyocyte specific (*Myh6, Cav1.2, CX43, MLC2v, cTnT*) transcription factor genes. Primer sequences can be furnished upon request. Two stage PCR was performed with each sample cDNA/primer pair done in triplicate. Relative quantification compared to control was estimated using the threshold cycle (CT) of the transcription factor normalized to the CT of the housekeeping gene *Gapdh*. Dissociation curve and ethidium bromide gel analysis was used to assess PCR product purity at the end of each qPCR run. Graphs are plotted as Relative Quantification Values or ratio to GAPDH.

2.3. Cell lineage analysis

To confirm the second heart field origin of jaw muscles we crossed *Isl1-Cre* mice (Yang et al., 2006) to the reporter line *mT/mG* (Jackson labs) (Muzumdar et al., 2007) to generate *Isl1-Cre;mT/mG* embryos. E13.5 embryos were collected by perfusion with variable fixation procedure described previously (Daughters et al., 2001). Embryos were embedded in OCT medium and frontal sections through the head region were collected on slides. Slides were visualized for GFP and dTomato staining and further processed for MHC (MF20; DSHB) or Pax7 (DSHB) immunostaining.

For lineage labeling of satellite cells we bred *Pax7-CreER* mice (Lepper et al., 2009) with *mT/mG* mice. *Pax7-CreER;mT/mG* mice were injected with Tamoxifen (3×5 mg at 3 day intervals) prior to isolation of satellite cells from the masseter and digastric muscles of the head. Satellite cell derived myoblasts were induced to form cardiomyocytes according the above differentiation scheme. Beating aggregates of induced cardiomyocytes were re-plated on glass culture slides and immunostained for cTnT (CT-3; DSHB) using a far-red tagged (Cy5) secondary antibody and visualized for co-localization with GFP.

For studies on the contribution of *Isl1* lineage derived satellite cells, jaw derived satellite cells were isolated from *Isl1-Cre;mTmG* mice generated from crossing *Isl1-Cre* to the reporter *mT/mG* (Jackson labs). For the *Isl1* derived satellite cells experiments, satellite cells were isolated from the masseter muscle of four mice per biological sample by collagenase/dispase digestion. *Isl1* lineage derived GFP^+ cells were sorted to obtain 100% positive expressing cells using the FACS Aria (BD). Cells were then subjected to the cardiomyocyte differentiation scheme and assayed for colocalization of NKX2.5 expression at day 7, or cTNT expression at day 14, with GFP.

2.4. Immunofluorescence and microscopy

Cell culture slides were fixed with 2% paraformaldehyde (PFA) (pH 8.5) for 15 min at room temperature and stored in 1X phosphate buffered saline A (PBSA) at 4 °C until processing. *Isl1Cre;mT/mG* mouse embryos were fixed using a variable pH PFA fixative procedure modified from (Daughters et al., 2001) overnight at 4 °C, washed in 1X PBSA and hardened overnight in 30% sucrose at 4 °C. The following day embryos were embedded in OCT (optimal cutting temperature) medium over dry ice and stored at −80 °C until processing. Embryos were processed by collecting 10 μm thick frontal sections through the head. Both cell culture slides and embryos were processed for expression of markers of myogenesis, cardiogenesis or mature cardiomyocytes. Briefly, slides were washed in 1x PBSA, permeabalized in PBSA containing 0.1% Triton, blocked in 5% normal goat serum (NGS) for 2 h at RT and incubated overnight with primary antibodies to either PAX7 (1/1000; Developmental Studies Hybridoma Bank, DSHB), MHC (MF20; 1/500; DSHB), NKX2.5 (1/200; Santa Cruz), GATA4 (1/500; ABCAM), Myogenin (F5D, 1:100; DSHB), cTnT (CT-3; 1/500; DSHB) in 5% NGS at 4 °C. The next day slides were washed 3×1 h in PBSA, blocked in 5%NGS for 1 h and incubated overnight in the appropriate secondary antibodies: Alexa-594 anti-rabbit, Alexa-488 anti-mouse or Alexa-634 anti-mouse (1/2000; Invitrogen) at 4 °C. Slides were mounted using Vectashield mounting medium containing DAPI (Vector labs) and visualized on a Fluoview 1000 confocal microscope with FV1000 analysis software (Olympus). Photomicrographs are composite images of 1–5 μm optical slices through the tissue compressed along the Z-axis. Images for figures were further processed using Photoshop (Adobe Systems) by cropping and by appropriate uniform and linear adjustments of brightness and contrast.

2.5. Electrophysiology

For electrophysiological recordings, induced cardiomyocytes were isolated from cultures on or after day 14 by picking or gentle dissociation with 0.1% collagenase, plated on glass culture slides, and covered in a perfusion chamber. Whole cell current clamp recordings were obtained from cells that were continuously superfused with solution containing 146 mM NaCl, 3 mM KCl, 10 mM HEPES, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 1 mM Na pyruvate, and 10 mM D-glucose (pH 7.4, NaOH). Patch pipettes contained 140 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM EGTA, 5 mM HEPES, 1 mM glutathione, 3 mM ATP-2K, 2 mM glucose, 0.5 mM GTP-Na (pH 7.2, KOH). Recordings were made at room temperature using pipettes with resistances ranging from 2 to 5 MΩ, a Multiclamp 700 A amplifier, Digidata 1322 A, and pClamp 9.2 acquisition software (Molecular