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Assessment of embryonic myocardial cell differentiation using a dual fluorescent reporter system

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Abstract

Recent studies have identified the existence of undifferentiated myocardial cells during early embryonic as well as post-natal stages of heart development. While primitive cells present in the precardiac mesoderm can differentiate into multiple cell types of the cardiovascular system, the developmental potential of undifferentiated cells identified in the ventricular myocardium after chamber formation is not well characterized. A deeper understanding of mechanisms regulating myocardial cell differentiation will provide further insights into the normal and pathological aspects of heart development. Here, we showed that Nkx2.5 positive and sarcomeric myosin negative cells were predominantly localized in the right ventricular myocardium of CD1 mice at E11.5 stage. We confirmed that myocardial regions negative for saromeric myosin were also devoid of atrial natriuretic factor (ANF). These observations are consistent with our previous study, which showed that ANF expression is restricted to moderately differentiated and mature myocardial cells in E11.5 myocardium of C3H/FeJ mice. Further, we found that the receptor c-Kit, a marker for early embryonic myocardial progenitor cells, is not expressed in the undifferentiated cells of the E11.5 myocardium. To monitor the differentiation potential of $Nkx2.5^{+}/ANF^{-}$ cells *in vitro*, we developed a novel double fluorescent reporter system. Subsequently, we confirmed that the majority of $Nkx2.5^{+}/ANF^{-}$ cells expressed mature myocyte markers such as sarcomeric myosin, MLC2V and alpha-cardiac actin after 48 hrs in culture, albeit at lower levels compared to $Nkx2.5^{+}/ANF^{+}$ or $Nkx2.5^{-}/ANF^{+}$ cell populations. Our results suggest that fluorescent reporters under the control of lineage-specific promoters can be used to study myocardial cell differentiation in response to various exogenous or pharmacological agents.

Keywords: embryonic heart • cell differentiation • development • fluorescent reporters

Introduction

During embryonic development, disturbances in the normal process of heart formation can result in congenital heart defects (CHD). In many of these cases, a combination of genetic and environmental factors is thought to play a critical role in the development of CHDs. Recent loss or gain of function genetic studies have shown that heart defects can occur either at the linear heart tube stage or after formation of a four-chambered structure in animal models [1]. For instance, ablation of the retinoid signalling pathway was shown to alter the ratio of undifferentiated to differentiated myocardial cells, promote precocious myocardial cell maturation and early embryonic lethality [2]. Similarly, elevated levels of homocystine and atrial natriuretic factor (ANF) in the amniotic fluid and maternal venous blood were shown to cause cardiac malformations in human foetuses [3–5]. Further characterization of cells and signals participating in myocardial cell differentiation post-chamber specification would enable the design of new therapeutic interventions and lower the early embryonic or peri-natal deaths associated with CHDs.

Although fate mapping and transgenic labelling studies have determined the precise location of early cardiac progenitor cells in several species [6], cell cycle dynamics, structural and functional attributes of these embryonic progenitor cells during cardiac ontogeny are not thoroughly characterized. Activation of early myocardial transcriptional factors such as Nkx2.5 is required for proper migration, lineage commitment and differentiation of cardiac progenitor cells [6]. Germ line ablation of Nkx2.5 has been shown to be associated with defects in early cardiac development

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[7]. It is widely accepted that embryonic cardiac progenitor cells differ from differentiated myocytes in that they do not express typical myocyte contractile proteins such as myosin heavy chain (MHC), myosin light chain (MLC) or secretory proteins such as atrial natriuretic factor (ANF) [8–10]. The ANF gene is considered as an excellent marker for the developing chamber myocardium during early stages of embryonic development [8]. We have recently shown that the ventricular myocardium of embryonic (E) day 11.5 heart harbours several undifferentiated cells that are positive for Nkx2.5 and negative for ANF expression [11]. Further, we found that both Nkx2.5 and ANF genes are expressed in differentiated cells of E11.5 myocardium in C3H/FeJ mice [11]. Consistent with our previous report, other studies have also documented the existence of undifferentiated myocardial cells in the mouse heart during E7.75–16.5 as well as post-natal stages of development [11–16]. While early cardiac progenitor cells identified in the cardiac crescent (E7.75) can give rise to all cardiac cell types (*e.g.* myocytes, epicardial, endocardial, vascular and conduction system cells) [6], progenitor cells isolated from E9.5 mouse hearts were shown to exhibit a more restricted differentiation potential (*e.g.* myocytes and vascular cells) [13]. However, the differentiation potential of the Nkx2.5 positive, ANF negative and sarcomeric myosin negative cells ($Nkx2.5^{+}/ANF^{-}sarcomere^{-}$) in the E11.5 myocardium is not well characterized [11].

In the present study, we showed that several Nkx2.5 positive and sarcomeric myosin negative cells ($Nkx2.5^{+}/MF20^{-}$) were predominantly localized in the right ventricular myocardium of CD1 mice at the E11.5 stage. We confirmed that myocardial regions negative for saromeric myosin were also devoid of ANF. In addition, we found that several of these undifferentiated cells exhibited predominantly a cytoplasmic Nkx2.5 staining pattern. To compare the differentiation potential of $Nkx2.5^{+}/ANF^{-}$ and $Nkx2.5^{+}/ANF^{+}$ cells *in vitro*, we developed a novel double fluorescent reporter system. Subsequently, we confirmed that the majority of $Nkx2.5^{+}/ANF^{-}$ cells expressed mature myocyte markers such as sarcomeric MHC, MLC2V and alpha-cardiac actin after 48 hrs in culture.

Materials and methods

Experimental animals

Experiments were performed on E11.5 embryos derived from pregnant CD1 mice (Charles River Laboratories, Montreal, Canada). Mice were maintained on a 12-hrs light dark cycle and all procedures were approved by the Dalhousie University Committee on Laboratory Animal Care. Female mice were mated with males and noon time on the day when the copulation plug was found was designated as day E0.5.

Tissue collection and sectioning

Pregnant mice were anaesthetized using 2.5% isoflurane delivered *via* a SurgiVet system (Model 100, WI) and sacrificed by decapitation. Embryos were collected in phosphate buffer saline (PBS), cryoprotected in PBS containing 30% sucrose and frozen in Tissue-tek® O.C.T. (Optimum Cutting Temperature compound, Sakura Finetek, Tokyo, Japan). Coronal sections (10 μm) were obtained using a cryostat (CM3050S, Leica Microsystems, Ontario, Canada).

Immunofluorescence analyses of histological sections

Ten micron sections derived from embryos and dispersed cardiomyocyte cultures were fixed in methanol or acetone and blocked with 10% goat serum and 1% BSA in PBS for 1 hr. The specimens were incubated with antibodies for Nkx2.5 (sc-14033, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and MF20 (DSHB, University of Iowa) or ANF (#CBL66, Chemicon), followed by anti-rabbit and anti-mouse secondary antibodies conjugated to Alexa fluor 555 and 488 (Invitrogen, Carlsbad, CA, USA). For c-Kit immunostaining, an FITC-labelled rat monoclonal antibody (CL8936F, Cedarlane, Ontario, Canada) was used. Nuclei were stained with 10 μg/ml of Hoechst dye for 5 min. Samples were examined using a Leica DM2500 fluorescence microscope or Zeiss LSM 510 Meta confocal microscope.

Gene constructions

To generate the Nkx2.5p-EGFP-SVpA construct, –2974 to +268 of the Nkx2.5 gene [17] was amplified from the mouse genomic DNA using specific primers and inserted upstream of the EGFP sequence (Clontech) followed by the SV40polyA sequence. To generate the ANFp-DsRed-prot.pA construct, the human ANF promoter [-500 to +77] [18] was amplified and inserted upstream of a nuclear localized DsRed2 sequence (Clontech) followed by the mouse protamine I polyA. The Nkx2.5p-EGFP-ANFpDsRed (NEAD) construct was generated by cloning the ANFp-DsRed-prot.pA expression cassette into Nkx2.5p-EGFP-SV40pA plasmid (Fig. 3A). Primer sequences are available upon request.

Myocardial cell cultures, gene transfer and reporter gene analysis

Embryonic hearts were dissected using a stereomicroscope (MZ16F, Leica). For whole ventricular cell preparations, atria and outflow tract (OFT) were carefully removed from the ventricles. For right ventricular cell preparations, hearts were visualized using a 1.6X Planapo Objective, left ventricular tissue was microdissected away from the bulbar groove to the apical region. Cells were isolated from ventricles by digestion with 0.2% type I collagenase (Worthington, NJ, USA) and counted using a haemocytometer. Approximately 3×10^{6} cells were plated on fibronectin-coated dishes in 10% FBS-DMEM. Cells were transfected with 2.5–4 μg of plasmid DNA using a Lipofectamine reagent (Invitrogen) for 6 hrs, fed with fresh medium and maintained for a further 48 hrs. Cells were fixed in a 4% paraformaldehyde buffer and reporter expression was visualized using appropriate filter sets. Transfection efficiency typically ranged from 25% to 30%.

FACS sorting and RT-PCR analyses

Cells transfected with the NEAD construct were sorted for fractions expressing EGFP and DsRed reporters using a FACS Calibur (Becton Dikinson). Sorted fractions were centrifuged at 3000 rpm to collect the cell pellet and

Table 1: Primers used for RT-PCR analysis

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
1) MLC2V	GCCAAGAAGCGGATA-GAAGG	CTGTGGTTCAGGGCTCAGTC
2) α Cardiac actin	GGATTCTGGCGATGGT-GTAA	CTCGTTGCCAATGGTGATGAC
3) Nkx2.5	CCCAAGTGCTCTCCT-GCTTTC	ATCTTGACCTGCGTGGACGT-GAGC
4) ANF	CGTGCCCCGACCCACG-CCAGCATGGGCTCC	GGCTCCGAGGGCCAGC-GAGCAGAGCCCTCA
5) 18S	TTGTTGGTTTTCG-GAACTGAGG	CATCGTTTATGGTCGGAAC-TACG

Note: All PCR primers were designed to span intron-exon junctions.

total RNA was extracted using a Trizol reagent (Invitrogen). Total RNA (0.1 μg) was reverse transcribed for 50 min at 42°C using Superscript II reverse transcriptase (RT, Invitrogen). Marker gene detection was performed by PCR using one-tenth of the RT reaction. RT-PCR primers sequences are provided in Table 1. PCR reactions were performed for 30 cycles: 30 sec. at 94°C, 30 sec. at 55°C, 60 sec. at 72°C.

Correlation of reporter gene expression and sarcomeric myosin staining

Cells were plated on etched grid coverslips (Belco), transfected with the NEAD construct and were visualized live using a Zeiss Axiovert microscope. Cell locations and fluorescence expression profiles were captured using a Zeiss Axiocam HRc camera. Subsequently, cells were immediately fixed in acetone and processed for MF20 immune reactivity, as described earlier. After processing, the tracked cells were located using the alphanumeric grid and scored for the MF20 reactivity.

Statistical analysis

Data are presented as mean ± S.E.M. Between-group comparisons were analysed by ANOVA and Tukey multiple comparisons test. Significance was assigned at $P < 0.05$. Analyses were performed using Graphpad Prism 4 software.

Results

Heterogeneous expression of Nkx2.5 and sarcomeric myosin proteins in the embryonic ventricular myocardium of CD1 mice

Expression profiles of Nkx2.5 and sarcomeric myosin proteins were assessed in E11.5 ventricular myocardium by immunofluorescence labelling. Both proteins were predominantly localized in the compact and trabecular zones of the ventricular myocardium (Fig. 1A–C). The right ventricular region flanking the OFT and atrioventricular cushion tissue (AVC) revealed a strong anti-Nkx2.5 but not sarcomeric myosin immunoreactivity (Fig. 1A–E). In some of these cells, the Nkx2.5 protein was observed only in the cytoplasm, while this protein was present in both nuclear and cytoplasmic compartments of other cells (Figs. 1E and 2D). Similarly, some of the myocardial cells present in the developing trabelculae of both ventricles were positive for Nkx2.5 but not MF20 immunoreactivity (Fig. 1E, arrowhead). Specificity of the immunostaining for both markers was confirmed by omitting primary antibodies in control samples (Fig. 1D).

c-Kit, a marker for early embryonic myocardial progenitor cells is not expressed in E11.5 ventricular myocardium

c-Kit is a transmembrane tyrosine kinase receptor specific for the Kit ligand [19]. This receptor was shown to be preferentially expressed in a bipotential myocardial and smooth muscle precursor cell population of the developing mouse heart [13]. To determine whether c-Kit receptor expression can be localized in E11.5 right ventricular regions positive for Nkx2.5 but devoid of sarcomeric myosin, we performed immunolabelling on cryosections using c-Kit antibodies. However, we did not observe any c-Kit positive cells either in the right ventricle or in the remainder of the heart (Fig. 1F). As a positive control, we assessed c-Kit expression in extra-cardiac regions of the E11.5 embryo. We observed membrane-specific staining of the c-Kit receptor in the head mesenchyme over the hindbrain and also within the lateral mesenchyme of the trunk regions (Fig. 1G). Specificity of the c-Kit receptor immunostaining was confirmed by omitting primary antibody in control samples (not shown).

Heterogeneous distribution of Nkx2.5 and ANF proteins in E11.5 ventricular cells derived from the CD1 mouse embryos

Expression profiles of Nkx2.5 and ANF proteins were analysed in cryosections by immunofluorescence analysis. The right ventricular $MF20^{-}$ region flanking the OFT and AVC was devoid of ANF staining but revealed a strong Nkx2.5 staining (Fig. 2A–D). Similarly, some regions of the compact myocardium of the right ventricle were also devoid of ANF but stained positive for Nkx2.5 immunoreactivity. Specificity of the immunostaining for both markers was confirmed by omitting primary antibodies in control samples. Collectively, our results suggest that the right ventricular myocardium flanking the OFT and AVC in E11.5 hearts is less differentiated than the remaining myocardium.

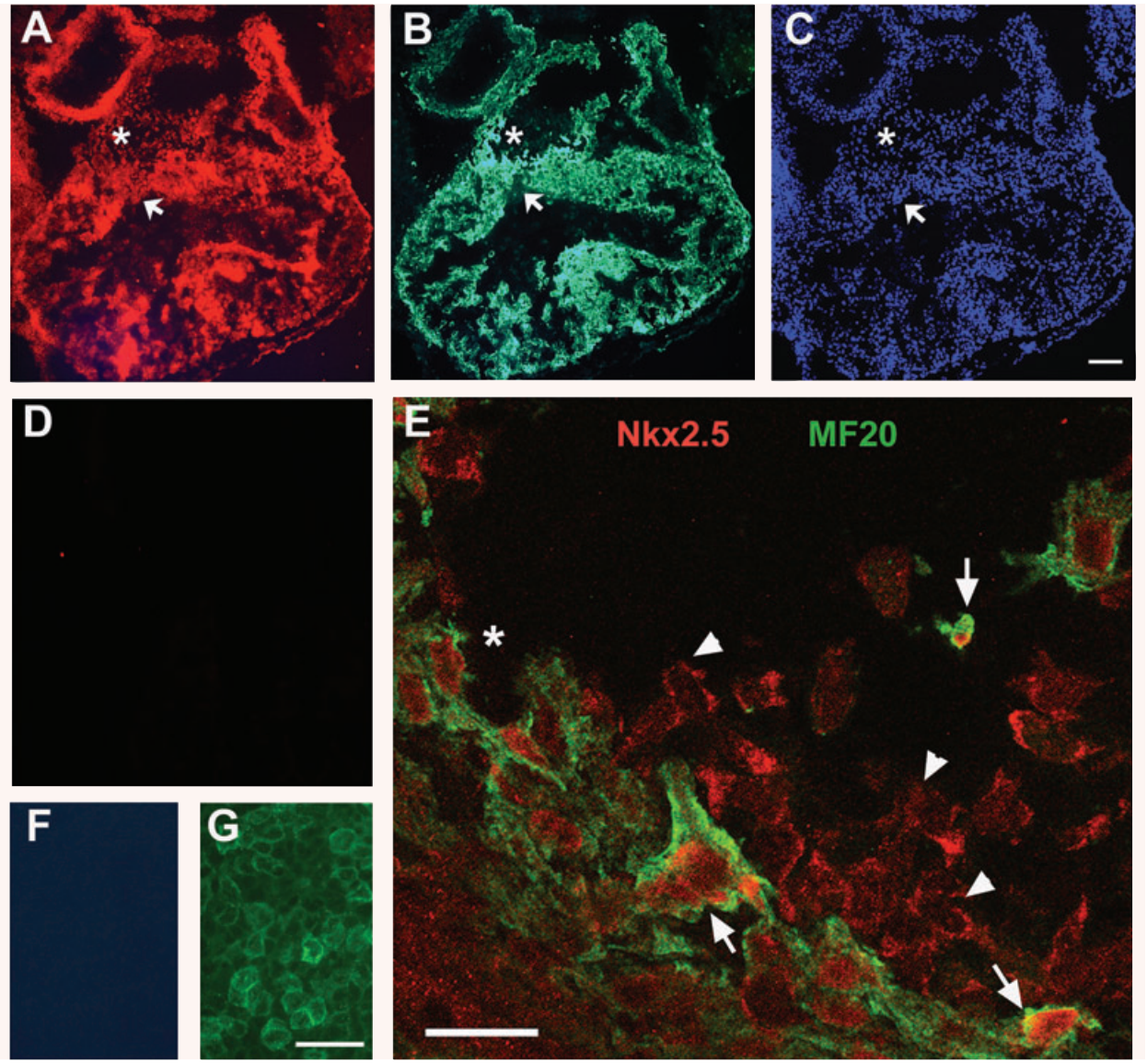

Fig. 1 (**A–E**) Identification of Nkx2.5^{+} and sarcomeric myosin negative cells in myocardium flanking AVC of E11.5 embryos. (**A–C**) Low power views of a section simultaneously processed for Nkx2.5 (**A**), MF20 (**B**) and nuclear stain (**C**). The location of AV cushion tissue is indicated by an asterisk and MF20 negative cells are indicated by an arrow. (**D**) Low power view of a control section incubated with secondary antibodies omitting primary antibodies. (**E**) Presence of cytoplasmic Nkx2.5 expression in cells negative for MF20 staining was confirmed by confocal microscopy (arrowheads). Cells positive for both Nkx2.5 and MF20 are indicated by arrows. (**F** and **G**) c-Kit receptor expression in the right ventricle (**F**) and head mesenchyme over the hind brain (**F**) of E11.5 embryos. Scale bars: 100 μm (**A–D**); 20 μm (**E**); 30 μm (**F** and **G**).

Generation and validation of double fluorescent reporter constructs for studies on right ventricular cell differentiation

We used the right ventricular specific murine Nkx2.5 promoter to target expression of an EGFP reporter and the human ANF promoter to target expression of a nuclear localized DsRed reporter to myocardial cells (Fig. 3A). E11.5 right ventricular myocardial cell cultures were transfected with the NEAD construct and processed after 48 hrs for simultaneous localization of EGFP, DsRed and nuclei using epifluorescence microscopy. In these cultures, we identified three distinct populations of myocardial cells: cells expressing only the EGFP reporter gene (Nkx2.5^{+}) (Fig. 2E–G), cells expressing only the

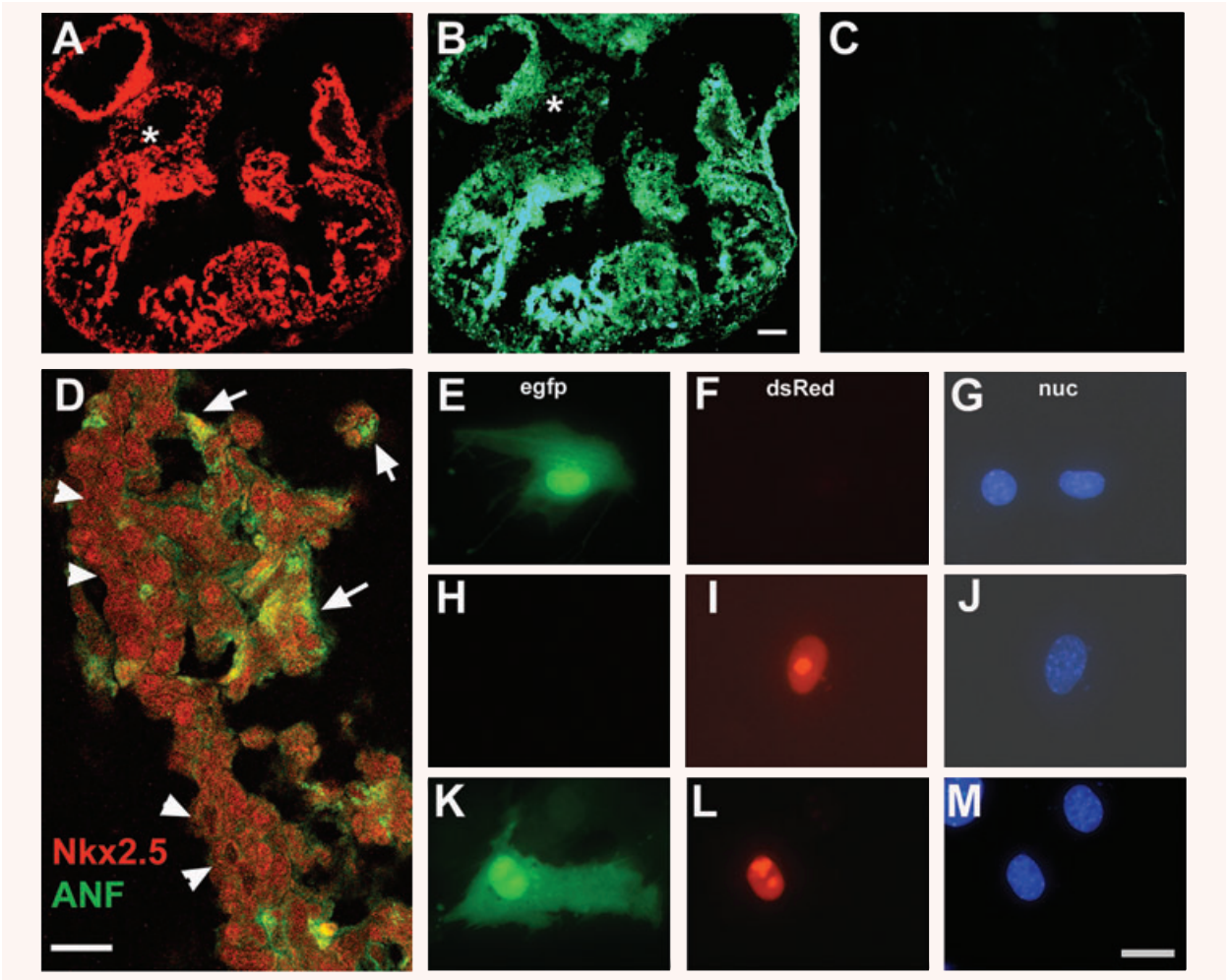

Fig. 2 (**A**–**F**) Identification of Nkx2.5$^+$ and ANF$^-$ cells in E11.5 myocardium. (**A**, **B**) Low power views of a section simultaneously processed for Nkx2.5 (**A**) and ANF (**B**) staining. (**C**) Low power view of a control section incubated with secondary antibodies omitting primary antibodies. (**D**) Absence of ANF expression and presence of Nkx2.5 staining in cells flanking AVC (arrowheads) was confirmed by confocal microscopy. Cells positive for both Nkx2.5 and ANF are indicated by arrows. The location of AVC is indicated by an asterisk. (**E**–**M**) Subcellular localization of EGFP (**E**, **H**, **K**), DsRed (**F, I, L**) and nuclear DNA (**G**, **J**, **M**) in E11.5 ventricular cells transfected with the NEAD transgene. (**E**–**G**) Nkx2.5$^+$/ANF$^-$ cell, (**H**–**J**) Nkx2.5$^-$/ANF$^+$ cell, (**K**–**M**) Nkx2.5$^+$/ANF$^+$ cell. Scale bars: 100 μm (**A**–**C**); 20 μm (**D**); 20 μm (**E**–**M**).

DsRed reporter gene (ANF$^+$, Fig. 2H–J) and cells positive for both EGFP and DsRed reporter expression (Nkx2.5$^+$/ANF$^+$, Fig. 2K–M). Overexpression of these reporter genes did not lead to overt cytotoxic effects in transfected cells as confirmed by counterstaining DNA with Hoechst 33342 (Fig. 2G, J, M). To determine the relative distribution of these three distinct cell populations, we scored the number of EGFP$^+$, DsRed$^+$ and EGFP$^+$DsRed$^+$ cells in random fields from the cultures transfected with the NEAD transgene. The results showed that approximately 29% of the transfected cells displayed only Nkx2.5 promoter activity while ~ 8% of the transfected cells were positive for only ANF promoter activity. In contrast, both promoters were active in ~ 63% of the transfected cells (Fig. 3B). For a comparison, we also transfected the NEAD construct in cells derived from the whole ventricle preparations (combined right and left ventricles). There was no significant difference between the percentages of cells identified using the NEAD construct between the right ventricle and those identified from whole ventricle preparations (Fig. 3B).

Fractionation and characterization of Nkx2.5$^+$/ANF$^-$, Nkx2.5$^+$/ANF$^+$ and Nkx2.5$^-$/ANF$^+$ myocardial cell populations *in vitro*

To compare the differentiation status of different cell populations, we fractionated cells expressing EGFP (Nkx2.5$^+$/ANF$^-$),

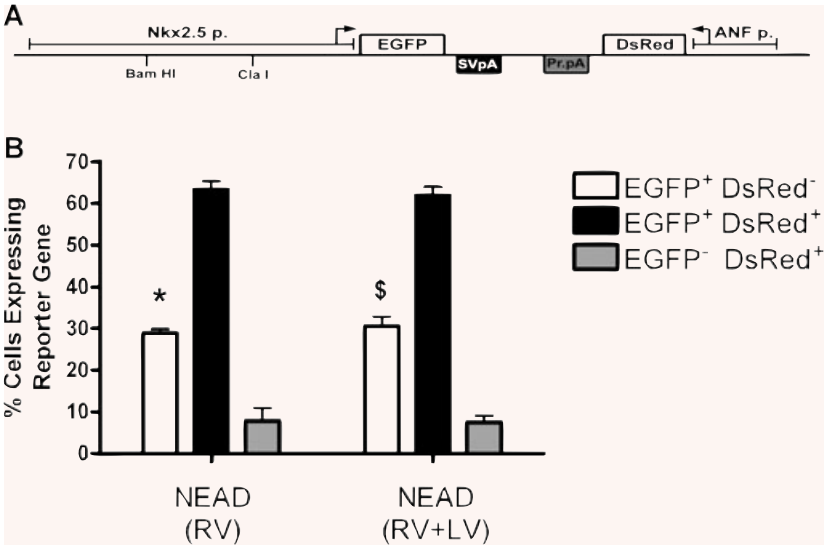

Fig. 3 (**A**) Schematic diagram of the transgenes used for *in vitro* tracking of cell differentiation, (**B**) Distribution of Nkx2.5$^+$/ANF$^-$ (EGFP$^+$), Nkx2.5$^+$/ANF$^+$ (EGFP$^+$/DsRed$^+$) and Nkx2.5$^-$/ANF$^+$ (DsRed$^+$) cells in E11.5 right (RV) or whole ventricular (RV+LV) cells transfected with the NEAD transgene. Data are presented as mean value ± S.E.M., n = 3–5 experiments. Overall, there was no significant difference in the frequency of three cell types between RV and RV+LV preparations. *P < 0.005, EGFP$^+$/DsRed$^-$ (RV) *versus* all other groups except EGFP$^+$/DsRed$^-$ (RV+LV) and $$P$ < 0.005, EGFP$^+$/DsRed$^-$ (RV+LV) *versus* all other groups except EGFP$^+$/DsRed$^-$ (RV).

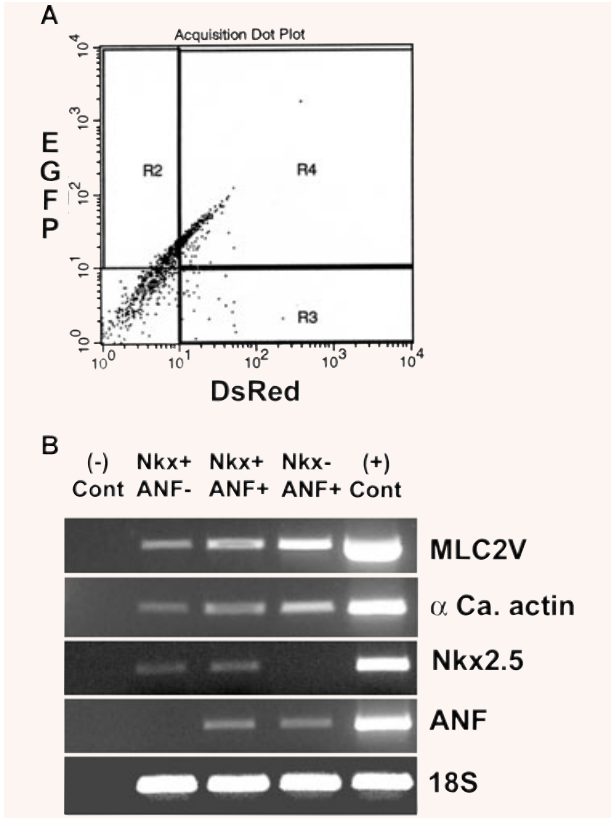

Fig. 4 *In vitro* characterization of Nkx2.5$^+$/ANF$^-$ (EGFP$^+$) and Nkx2.5$^+$/ANF$^+$ (EGFP$^+$/DsRed$^+$) myocardial cells. (**A**) FACS separation of EGFP$^+$/DsRed$^-$ EGFP$^+$/DsRed$^+$ and EGFP$^-$/DsRed$^+$ fractions from the NEAD transfected E11.5 ventricular cells, R: Region (**B**) RT-PCR analysis of FACS sorted cells for the expression of MLC2V, alpha-cardiac actin, Nkx2.5 and ANF.

EGFP and DsRed (Nkx2.5$^+$/ANF$^+$) or DsRed (Nkx2.5$^-$/ANF$^+$) reporter genes by FACS sorting (Fig. 4A). Total RNA isolated from these three cell types was processed for RT-PCR analysis of two myocyte differentiation markers, MLC2V and α-cardiac actin. In addition, RNA samples were also analysed for the endogenous transcript levels of Nkx2.5 and ANF genes. The results indicate that the Nkx2.5$^+$/ANF$^-$ cells harbour significantly lower levels of transcripts encoding MLC2V and α-cardiac actin compared to the levels seen in other two cell types (Fig. 4B). Further our results confirm that endogenous Nkx2.5 and ANF transcripts are appropriately expressed in FACS sorted cell populations (Fig. 4B).

In addition to the RNA analyses, we processed Nkx2.5$^+$/ANF$^-$ cells for the expression of mature myocardial markers at the protein level by immunocytochemistry. To this end, E11.5 ventricular cells were isolated and plated on grid etched coverslips and transfected with the NEAD construct. Forty-eight hours after transfection, cells expressing only EGFP were observed and their locations recorded. Cells were immediately fixed with acetone and then processed for sarcomeric myosin staining. Although EGFP fluorescence was lost using acetone fixation, we were able to determine cell locations after fixation using the alphanumeric numbering on the grids. The immunostaining results indicated that approximately 75% of Nkx2.5$^+$/ANF$^-$ cells were positive for MF20 staining while 25% were negative for sarcomeric myosin (Fig. 5).

Discussion

Several groups have documented the existence of undifferentiated cardiac progenitor cells during embryonic or post-natal stages of heart development [11–16]. In this study, we showed that the

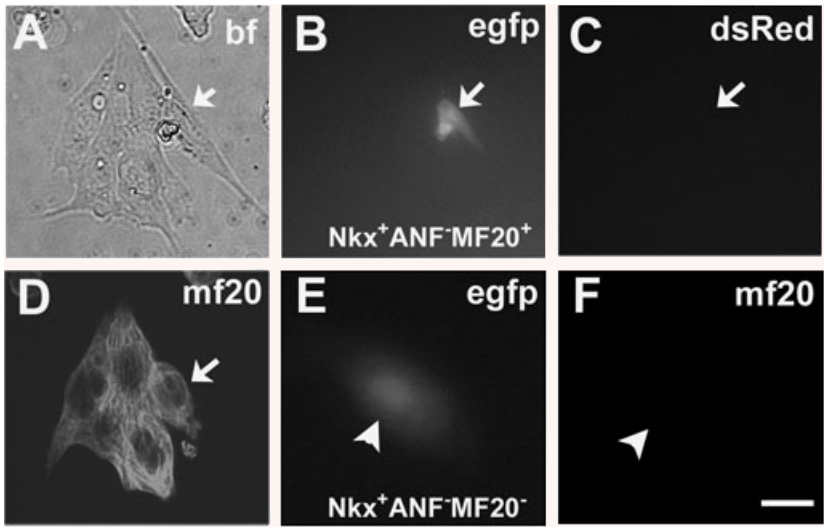

Fig. 5 Immunostaining analysis on NEAD transfected cells using sarcomeric myosin antibodies: (**A**–**D**) Micrograph of a Nkx2.5$^+$/ANF$^-$ cell expressing EGFP (**B**) but not DsRed (**C**) or sarcomeric myosin (**D**). (**E** and **F**) Micrograph of an Nkx2.5$^+$/ANF$^-$ cell expressing EGFP (**E**) but not sarcomeric myosin (**F**). Arrow and arrowhead indicate the position of the transfected cells in panels A–D and E and F, respectively. Scale bar: 20 μm (**A**–**F**).

Nkx2.5$^+$/ANF$^-$sarcomere$^-$ cells were predominantly localized in the right ventricular region flanking the OFT and AVC. Some of these undifferentiated cells showed an exclusive cytoplasmic localization of the transcription factor Nkx2.5. Phosphorylation of a conserved serine residue between the first and second helix of the Nkx2.5 homeodomain by casein kinase II was shown to play a vital role in the nuclear transport of this protein [20]. Although, the functional significance of cytoplasmic Nkx2.5 is not known, this may partially explain the delayed differentiation status of some myocardial cells identified in this study. A similar cytoplasmic localization of another cardiac transcription factor Tbx5 was reported in the cushion tissue of the OFT and AVC in the developing mouse heart [21].

It is likely that undifferentiated cells identified in our study are temporally and spatially distinct from the previously described cardiac progenitor cells and may be particularly relevant to the late myocardialization process of the right ventricle. Wu and colleagues fractionated a bipotential cardiac progenitor cell population positive for Nkx2.5 and the receptor tyrosine kinase, c-Kit from E9.5 mouse hearts [13]. In line with these studies, we tested whether the Nkx2.5$^+$/ANF$^-$/MF20$^-$ cells present in the myocardial region flanking the OFT and AVC were also positive for c-Kit immunoreactivity. However, we found that c-Kit expression was absent in the entire myocardium and was limited to the head and trunk mesenchyme cells at E11.5 stage. These results are in agreement with a previous report, which documented the presence of mRNA for c-Kit or its ligand only in the migrating neural crest cells but not in the myocardium of B6 mouse embryos at E11.5 stage [19].

To facilitate cell tracking and fate determination of Nkx2.5$^+$/ANF$^-$ cells in the right ventricle, we developed the NEAD fluorescent reporter system. The cell tracking ability of the NEAD reporter was not significantly different between cell preparations derived from the right ventricle or those derived from both ventricles. The relative percentages of different cell populations detected by the NEAD transgene in CD1 myocardium are in good agreement with those detected by immunogold labelling of C3H/FeJ ventricular myocardium of a comparable developmental stage. It was documented in earlier studies that ANF transcripts are down-regulated in the right ventricle prior to the left ventricle particularly in the region flanking the AVC at E11.5 stage of the FVB mouse strain [22]. In agreement with this study, we did not observe immunoreactive ANF protein in the right ventricular region flanking AVC in our study. Further, using anti-ANF immunogold labelling technique, we found that the ANF expression correlated well with the presence of sarcomeres in the right ventricular myocardial cells located distal to the AVC in our study (Zhang and Pasumarthi, data not shown).

Differentiation of Nkx2.5$^+$/ANF$^-$ cells from the right ventricle was confirmed *in vitro* using FACS sorted cell poupulations after 48 hrs in culture. Gene expression analysis indicated that Nkx2.5$^+$/ANF$^-$ cells contain low levels of transcripts coding for mature muscle markers MLC2v and α-cardiac actin compared to those of the Nkx2.5$^+$/ANF$^+$ or Nkx2.5$^-$/ANF$^+$ cell groups. The validity of cell sorting technique was further confirmed by the appropriate expression of endogenous Nkx2.5 and ANF transcripts in FACS sorted cell populations. At present, it is not known whether the absence of c-Kit receptor expression in E11.5 heart cells play any role in the delayed differentiation of myocardial cells. However, it is clear from the published studies that mutations in the gene coding for this receptor (*white spotting, W*) do not cause defects in the embryonic heart development [23, 24]. Lower levels of MLC2V and α-cardiac actin transcripts in Nkx2.5$^+$/ANF$^-$ cells also suggest that only a fraction of these cells may be starting to differentiate into a cardiomyocyte phenotype in culture. To more precisely assess cellular differentiation status of cultured Nkx2.5$^+$/ANF$^-$ cells at a single cell level, we investigated the expression of sarcomeric myosin (MF20) by immunocytochemistry. Our results indicated that approximately 25% of Nkx2.5$^+$/ANF$^-$ cells did not express sarcomeric myosin after 48 hrs in culture. Although, a triple immunolabelling approach with antibodies specific for Nkx2.5, ANF and MF20 would also address this question; such an approach was not feasible in this

study due to the fact that two monoclonal antibodies (MF20 and ANF) were of mouse origin. The fact that 75% of $Nkx2.5^{+}/ANF^{-}$ cells were positive for mature myocyte-specific markers after 48 hrs in culture clearly underscores a role for these cells in myocardialization of the right ventricle at the stage where remodelling of major blood vessel connections and valve formation are known to occur [25, 26]. Indeed, a distinct population of mesenchymal cells in E12 stage mouse hearts was shown to form differentiated myocardial cells along the OFT, AVC, pulmonary and caval veins [25, 26]. Current efforts in our laboratory are aimed at further characterization of the fate and Ca^{+2} handling properties of different subpopulations of right ventricular myocardial cells at a single cell level.

In conclusion, we showed that the majority of $Nkx2.5^{+}/ANF^{-}$ cells expressed mature myocyte markers such as sarcomeric MHC, MLC2V and alpha-cardiac actin after 48 hrs in culture, albeit at lower levels compared to $Nkx2.5^{+}/ANF^{+}$ or $Nkx2.5^{-}/ANF^{+}$ cell populations. In addition, we demonstrated that the fluorescent reporters under the control of lineage-specific promoters could be used to study embryonic myocardial cell differentiation. This method should be particularly useful for isolation and characterization of undifferentiated myocardial cells after chamber formation given the absence of well-established progenitor cell-specific markers such as c-Kit and Isl1. We also suggest that the newly developed dual fluorescence reporter system may be utilized to monitor the effects of various exogenous or pharmacological agents on cardiac cell differentiation in real-time.

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