

# Ultrastructural and immunocharacterization of undifferentiated myocardial cells in the developing mouse heart

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## Abstract

The recent discovery of several myogenic cardiac progenitor cells in the post-natal heart suggests that some myocardial cells may remain undifferentiated during embryonic development. In this study, we examined the subcellular characteristics of the embryonic (E) mouse ventricular myocardial cells using transmission electron microscopy (TEM). At the ultrastructural level, we identified three different cell populations within the myocardial layer of the E11.5 heart. These cells were designated as undifferentiated cells ( $43 \pm 6\%$ ), moderately differentiated cells ( $43 \pm 2\%$ ) and mature cardiomyocytes ( $14 \pm 4\%$ ). Undifferentiated cells contained a large nucleus and sparse cytoplasm with no myofibrillar bundles. Moderately differentiated cells contained randomly arranged myofilaments in the cytoplasm. In contrast, mature cardiomyocytes contained well-developed sarcomere structures. We also confirmed the presence of similar undifferentiated cells albeit at low levels in the E16.5 (~20%) and E18.5 (~7%) myocardium. Further we used immunogold labeling technique to test whether these distinct cell populations were also positive for markers such as Nkx2.5, ISL1 and ANF. A preponderance of anti-Nkx2.5 label was found in the undifferentiated and moderately differentiated cell types. Anti-ANF label was found only in the cytoplasmic compartment of moderately differentiated and mature myocardial cells. All of the undifferentiated cells were negative for anti-ANF labeling. We did not find immunogold labeling with ISL1 in any of the three myocardial cell types. Based on these results, we suggest that embryonic myocardial cell differentiation is a gradual process and undifferentiated cells expressing Nkx2.5 in post-chamber myocardium may represent a progenitor cell population while cells expressing Nkx2.5 and ANF represent differentiating myocytes.

**Keywords:** cardiac progenitor cells • routine TEM • immunogold labeling • Nkx2.5 • embryonic myocardium

## Introduction

The mammalian heart is formed from the mesodermal progenitor cells present in the primary heart field

(PHF) and the anterior heart field (AHF) of the developing embryo [1–5]. It is generally believed that these cardiac progenitor cells (CPCs) extensively proliferate and simultaneously differentiate into multiple cardiac cell types such as myocytes, epicardial and endocardial cells, vascular and conduction system cells [1]. The undifferentiated CPCs present in both heart fields are known to express unique markers such as the homeodomain containing transcription factor Nkx2.5, bHLH

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transcription factors *Mesp1* and *2* and the T-box protein *brachyury* [1, 6, 7]. While the LIM-domain containing transcription factor *islet 1 (ISL1)* is only expressed in the AHF, markers expressed exclusively in the PHF are yet to be identified [1]. Interestingly, the expression levels of transcription factors such as *Mesp*, and *ISL1* were shown to be down regulated in the later stages of cardiac development [7–9]. In contrast, *Nkx2.5* is expressed in both undifferentiated CPCs of the heart fields as well as in the differentiated myocytes later in the development [1, 10–12]. However, differentiated myocytes in the embryonic heart differ from the CPCs in that they express typical markers such as sarcomeric proteins (myosin heavy chain, actin, myosin light chains, etc.) and secretory proteins such as ANF [13–15].

Recent studies have identified several rare populations of CPCs in the postnatal myocardium [8, 16–20]. These new findings have raised the possibility that some of the PHF and AHF derived mesodermal cells may remain undifferentiated during embryonic heart development. However, there is no information available on the existence, fate and structural attributes of the CPCs in the embryonic heart post-chamber specification. In this study, we sought to determine whether the embryonic ventricular myocardium contains any undifferentiated cells expressing markers such as *Nkx2.5* or *ISL1* post-chamber specification. Using transmission electron microscopy (TEM), we have identified several undifferentiated cells that are positive for the earliest known cardiac marker *Nkx2.5* but not *ISL1* in the embryonic (E) day 11.5 ventricular myocardium. We also found a progressive decrease in the undifferentiated cell pool and a concomitant increase in the number of mature cardiomyocytes during heart development.

## Materials and methods

### Experimental animals

Experiments were performed on the heart tissue isolated from different stages of mouse embryos. The initial breeding pair of C3H/FeJ mouse strain was obtained from the Jackson Laboratory (Bar Harbor, ME). The breeding colony was maintained in-house and female mice were mated with males under a 12 hr light/dark cycle. The noon time on the day when the copulation plug was found was designated as embryonic

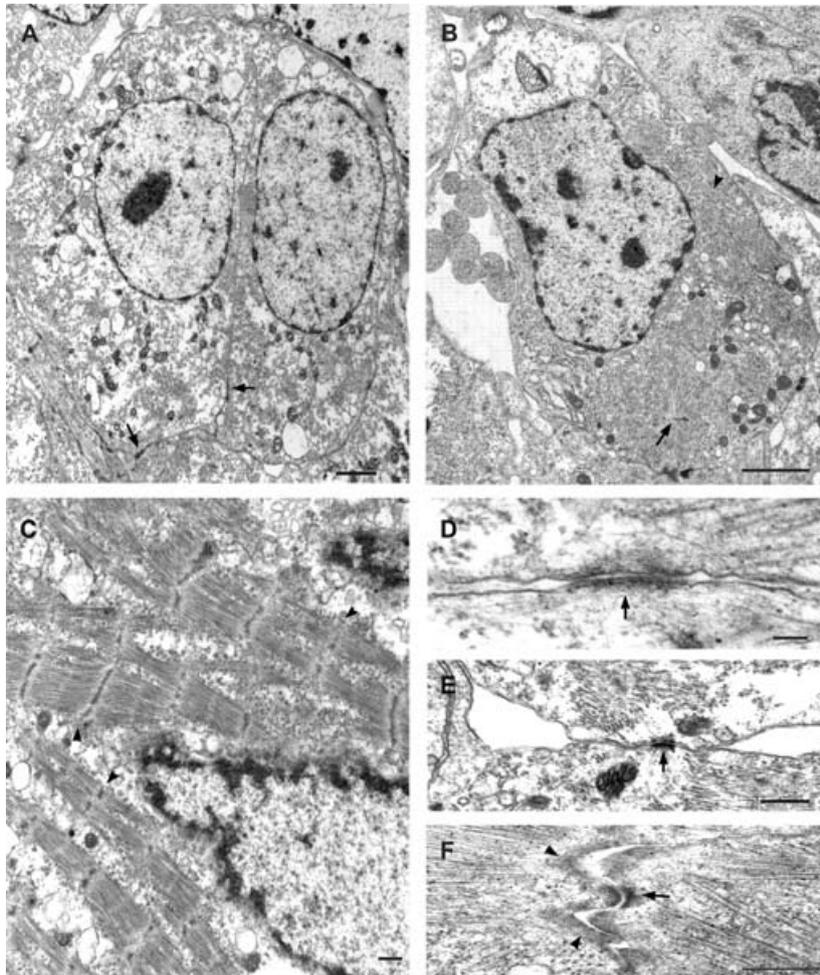
0.5 day post coitus (p.c). For the collection of tissue specimens, pregnant mice were sacrificed, embryos were isolated from the uterine horns and the embryonic hearts were dissected using a stereomicroscope. Atrial appendages from all these samples were carefully removed and only ventricular tissue was processed for embedding. All of these procedures were performed according to the guidelines set by the Canadian Council on Animal Care and were approved by the Dalhousie University Committee on Laboratory Animal Care.

### Routine TEM method

Ventricular specimens isolated from the embryonic hearts were fixed in 2.5% glutaraldehyde (Canemco-Marivac, Quebec) in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C for 2 hrs, post-fixed in 1% osmium tetroxide (Canemco-Marivac) for 2 hrs and placed in 0.25% uranyl acetate (Canemco-Marivac) at 4°C overnight. Tissue specimens were dehydrated in graded series of acetone and embedded in Epon Araldite resin (Canemco-Marivac) and polymerized for 48 hrs at 60°C. Ultrathin sections (~80 nm) were cut using an ultramicrotome (LKB Huxley, England), placed on copper grids and stained with uranyl acetate and lead citrate. Samples were examined using a JEOL JEM 1230 transmission electron microscope (TEM) and images were captured using a Hamamatsu camera.

### Immunogold labeling technique

Ventricular specimens were fixed overnight with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer and dehydrated in a graded series of ethanol. After embedding in LR White resin (Canemco-Marivac), ultrathin (~80 nm) sections were cut, placed on nickel grids, washed in sodium borohydride followed by 30 mM glycine in 0.1 M borate buffer (pH 9.6), blocked in Tris-buffered saline (TBS, pH 8.1) containing 1% skim milk and 1% BSA for 45 min. and incubated with primary antibodies against *Nkx2.5* (sc-14033, Santa Cruz Biotechnology Inc.), ANF (#CBL66, Chemicon) or *ISL1* (Clone 39.4D5, DSHB, University of Iowa) overnight at room temperature. Sections were subsequently incubated with anti-mouse IgG antibodies coupled to 5 nm gold particles (Sigma, Ontario) or anti-rabbit IgG antibodies coupled to 10 nm gold particles (Sigma) for 1 hr, washed with TBS, post-fixed in 2.5% glutaraldehyde, rinsed and counterstained with uranyl acetate and lead citrate. For double immunogold labeling, the grids were incubated first with *Nkx2.5* antibodies followed by anti-rabbit IgG secondary antibodies and subsequently with ANF antibodies followed by anti-mouse IgG secondary antibodies. For controls, primary antibodies were omitted from the procedure.



**Fig. 1** Ultrastructure analysis of E 11.5 ventricular myocardium. (A) Two adjacent undifferentiated cells with sparse cytoplasm: note the presence of belt desmosomes between these two cells (arrows), Scale bar = 2  $\mu$ m. (B) A moderately differentiated myocardial cell with loosely arranged myofilaments (arrowhead) and a primitive sarcomere (arrow) in the cytoplasm, Scale bar = 2  $\mu$ m. (C) A mature myocardial cell containing myofilament bundles with well-defined sarcomeres (arrowheads), Scale bar = 500 nm. (D) A belt desmosome (arrow) between two undifferentiated cells, Scale bar = 100 nm. (E) A spot desmosome (arrow) between moderately differentiated cells, Scale bar = 500 nm. (F) An intercalated disc between two mature myocardial cells: fascia adherens (arrowheads) and desmosome (arrow). Scale bar = 500 nm.

## Statistical analysis

Data are presented as mean  $\pm$  S.E.M. Between-group comparisons were analysed by ANOVA multiple comparisons test (Graphpad Insat 3). Significance was assumed at  $P < 0.05$ .

## Results

### TEM analysis of myocardial cells in the E11.5 ventricular myocardium

At day E 11.5, the right and left chambers of developing ventricles are separated by a primitive septum and the ventricular wall is composed of an outer layer of epicardial cells, a myocardial cell layer and an inner layer of endocardial cells [21]. To investigate whether there are any undifferentiated cells in the embryonic

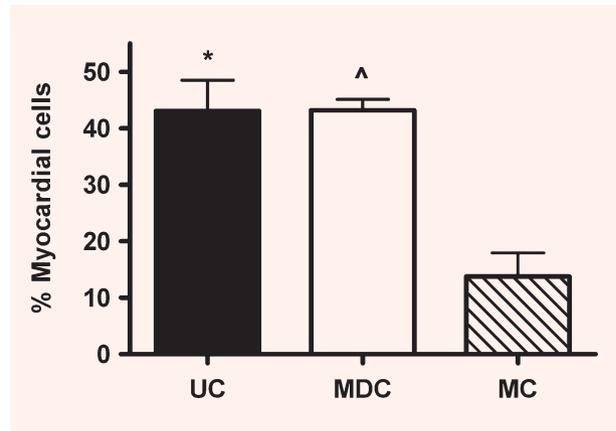
hearts, we examined subcellular characteristics of E 11.5 ventricular myocardial cells using the routine TEM. At the ultrastructural level, we observed three different cell populations within the myocardial layer which include small undifferentiated cells, moderately differentiated cells and mature cardiomyocytes (Fig. 1A–C). These cells were clearly distinguishable from the epithelial cells, endothelial cells, blood cells, interstitial cells, nerve fibers and other non-muscle cell types by their distinct ultrastructural characteristics. Each of the smaller undifferentiated cells contained a large nucleus and very sparse cytoplasm. The majority of the nuclear chromatin in these cells appeared as clumps lining immediately below the nuclear envelope and the nucleoplasm contained only a few blocks of chromatin. The cytoplasmic compartment of these cells lacked myofibrillar bundles and contained fewer organelles such as mitochondria, Golgi apparatus,

endoplasmic reticulum (ER) and small vesicles (Fig. 1A). Undifferentiated cells were frequently found in close proximity to the endocardial cell layer. Many of these undifferentiated cells formed cell to cell junctions with adjacent undifferentiated or moderately differentiated and mature myocardial cells *via* spot or belt desmosomes (Fig. 1D).

In addition to undifferentiated cells, the myocardial layer in E11.5 ventricle was also composed of several mature cardiomyocytes exhibiting well-developed sarcomere structures as well as moderately differentiated cells at varying stages of differentiation. Both moderately differentiated and mature cell types contained abundant cytoplasm and a large nucleus with several blocks of clumped chromatin dispersed in the nucleoplasm (Fig. 1B and C). The cytoplasmic compartment of moderately differentiated cells contained randomly arranged myofilaments lacking a well-organized sarcomeric structure, organelles such as mitochondria, Golgi, ER and small vesicles as well as secretory granules (Fig. 1B). In contrast, the cytoplasmic compartment of mature cells contained several myofilament bundles arranged into well-organized sarcomeric structures, abundant mitochondria, secretory granules as well as other organelles (Fig. 1C). Both moderately differentiated and mature myocardial cell types formed cell to cell junctions with each other mainly *via* desmosomes and fascia adherens (Fig. 1E). Intercalated discs containing gap junctions, desmosomes and fascia adherens were also observed in many of the differentiated cells (Fig. 1F).

### Quantitation of undifferentiated and mature myocardial cells in the developing ventricular myocardium

Based on the ultrastructural characteristics described earlier, we assessed the percentages of undifferentiated, moderately differentiated and differentiated cells in the E11.5 ventricular myocardium (Fig. 2). We defined myocytes as moderately differentiated cells when the cytoplasm contained scattered myofilaments lacking organized sarcomeric structures. At E11.5, we found that the majority of ventricular myocardial cells were either undifferentiated or moderately differentiated. Interestingly, mature or differentiated myocytes represented only ~14% of the E11.5 ventricular myocardium. We subsequently



**Fig. 2** Relative distribution of undifferentiated (UC), moderately differentiated (MDC) and mature (MC) myocardial cells in E11.5 ventricular myocardial cell layer. Data is presented as mean value ± S.E.M.,  $N = A$  total of 1000 cells from five different hearts. \*  $^{\wedge}p < 0.005$  compared to MC.

confirmed the presence of these three types of cells and structural characters in the E16.5 and E 18.5 ventricular myocardium (Table 1). Quantitative analyses indicated a decrease in the undifferentiated cell pool (E11.5 = 43%, E16.5 = 20% and E18.5 = 7%) and an increase in the number of mature cardiomyocytes (E11.5 = 14%, E16.5 = 38%, E18.5 = 61%) during development (Table 1).

### Immunocharacterization of undifferentiated and mature myocardial cells in the E11.5 ventricular myocardium

To test whether these distinct cell populations identified by the routine TEM were also positive for markers such as Nkx 2.5, ISL1 and ANF, immunogold staining technique was used. At E11.5 stage, a preponderance of anti-Nkx 2.5 label was found in the undifferentiated and moderately differentiated cell types and was distributed evenly between the nuclear and cytoplasmic compartments (Figs 3C and 4A). We also found low levels of Nkx2.5 labeling in mature cardiomyocytes (Fig. 4B). In contrast, the anti-ANF label was only found in the cytoplasmic compartment of moderately differentiated and mature myocardial cells (Fig. 4C and D). A unique feature of the anti-ANF labeling was that the majority of signal was clearly associated with

**Table 1** Relative distribution of three myocardial cell types in the ventricular myocardium during development

Developmental stage	Undifferentiated cells (UC)	Moderately differentiated cells (MDC)	Mature cardiomyocytes (MC)
E11.5	43.0 ± 5.5%*	43.0 ± 2.0%	14.0 ± 4.2%** , #
E16.5	20.0 ± 2.1%^	42.0 ± 2.3%	38.0 ± 1.0%
E18.5	7.0 ± 0.3%\$	32 ± 0.4%^^	61.0 ± 0.7%

Data is presented as mean ± S.E.M., *N* = A total of 600–1000 cells from three to five hearts per group.

\**p* < 0.05 E11.5 UC *versus* E16.5 UC and *p* < 0.005 E11.5 UC *versus* E18.5 UC.

\*\**p* < 0.005 E11.5 MC *versus* E11.5 UC and E11.5 MDC.

#*p* < 0.05 E11.5 MC *versus* E16.5 MC and *p* < 0.005 E11.5 MC *versus* E18.5 MC.

^<0.05 E16.5 UC *versus* E16.5 MDC.

\$<0.05 E18.5 UC *versus* E18.5 MDC and *p* < 0.005 E18.5 UC *versus* E18.5 MC.

^^<0.005 E18.5 MDC *versus* E18.5 MC.

clusters of secretory granules. As anticipated, all the undifferentiated cells were negative for anti-ANF labeling (Fig. 3B). In addition, we did not find immunogold labeling with ISL1 in any of these myocardial cell types (data not shown).

To further substantiate these results, we used a double immunogold staining technique and simultaneously assessed the percentages of myocardial cells expressing Nkx2.5 and or ANF markers in the E11.5 hearts. In agreement with ultrastructural features and single immunogold staining results, double staining revealed that all of the undifferentiated myocardial cells were positive for only anti-Nkx2.5 label while differentiated myocardial cells were positive for both anti-Nkx 2.5 and ANF labels (Fig. 5A). Based on the double immunogold labeling experiments on three independent E11.5 ventricles, undifferentiated cells positive for Nkx2.5 represented approximately 36% of the total cells counted (Fig. 5B). In addition, the majority of myocardial cells (~59%) were positive for both Nkx2.5 and ANF markers, while about 5% of the myocardial cells were positive for only ANF (Fig. 5B). As anticipated, the later two groups of immunogold positive cells (Nkx2.5+ANF or ANF only positive) comprised of both moderately differentiated and mature myocardial cells but not undifferentiated cells. Collectively, our results indicate that a large number of myocardial cells expressing the transcription factor Nkx 2.5 remain undifferentiated in the embryonic heart post-chamber specification.

## Discussion

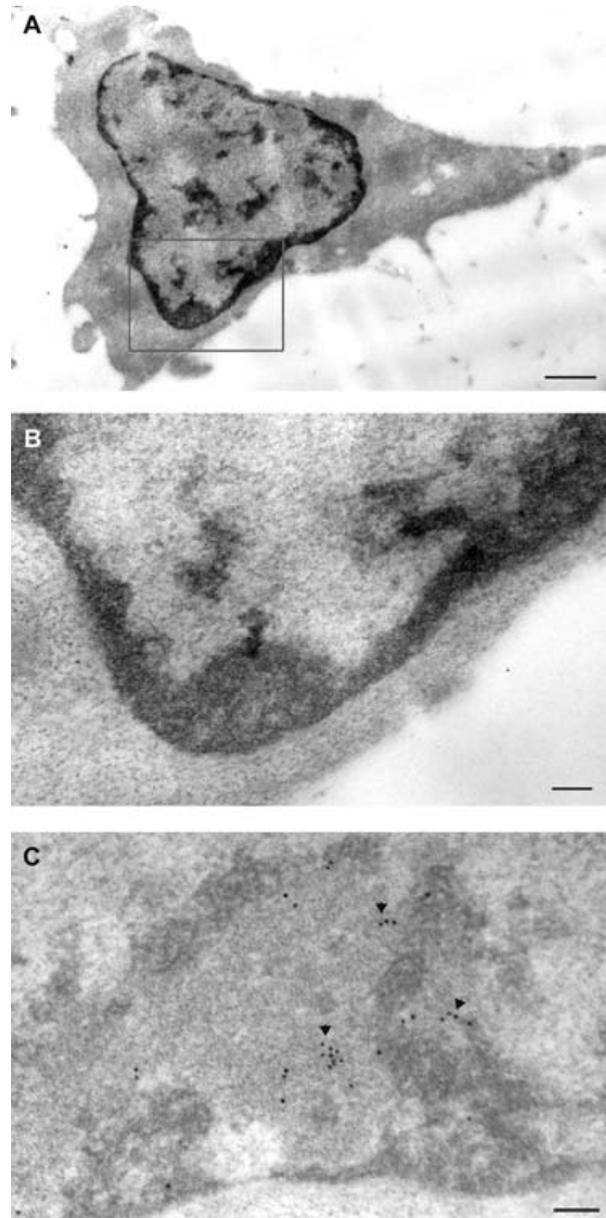
Although various steps involved in the morphogenetic transformation of the embryonic heart are clearly defined [13, 22], there is no spatiotemporal information on the differentiation status of the cardiogenic mesodermal cells that are mainly responsible for the formation of the heart. In the present study, we studied the ultrastructural properties of different cell populations within the myocardial layer of the E 11.5 heart using the routine TEM technique. Our results suggest that the bulk of myocardial cells remain undifferentiated or partially differentiated in the embryonic heart immediately after chamber specification. Based on ultrastructural features, these cells were clearly distinct from other non muscle cell types including smooth muscle cells [23] and recently discovered interstitial cajal-like cells [24–26].

To further characterize embryonic myocardial cells, we used the immunogold labeling technique and assessed the expression of independent markers such as Nkx2.5, ISL1 and ANF. We found that all of the undifferentiated cells in the E11.5 myocardium expressed the transcription factor Nkx2.5 but not ISL1 and ANF (Fig. 3). These undifferentiated cells differ from other non-cardiac cell types based on their unique morphology. Since cardiac fibroblasts were shown to be devoid of Nkx2.5 expression [12, 27], undifferentiated cells identified in this study

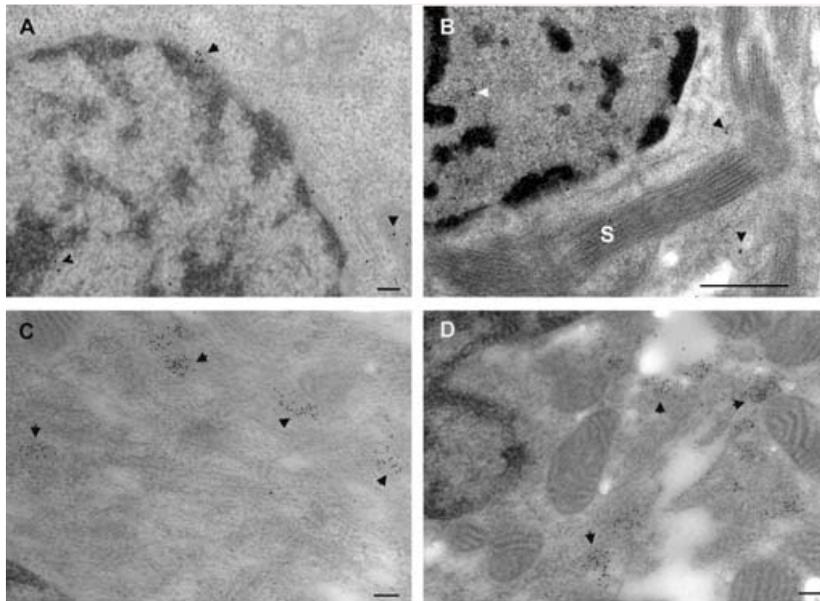
can be easily ruled out as putative fibroblast population. Moreover, expression of Nkx2.5 in undifferentiated cells clearly suggests that not all the PHF and AHF derived cells are simultaneously differentiated in the developing heart. In contrast, moderately differentiated and mature cardiomyocytes did not express ISL1, but were positive for ANF and Nkx2.5 expression.

Furthermore, our double immunogold labeling experiments also revealed ANF expression in moderately differentiated and mature cardiomyocytes but not in Nkx2.5 positive undifferentiated cells (Fig. 5B). Although there was a discrepancy between the relative percentages of undifferentiated cells identified *via* double immunogold labeling (~36%, Fig. 5B) and those identified via routine TEM (~43%, Fig. 2), this was not due to a real heterogeneity of undifferentiated cells since all of them expressed only Nkx2.5. Moreover, this difference is not statistically significant and thus can be attributed to individual animal or experimental variations. Surprisingly, a small percentage of myocardial cells positive only for ANF were also found in E11.5 myocardium (Fig. 5B). These ANF positive cells could be resulting from the non-Nkx2.5 positive CPCs that were shown to form venous pole of the developing heart [28]. Alternatively, Nkx2.5 expression in these cells may be developmentally downregulated. Certainly, downregulation of transcription factors such as GATA4 that are known to control Nkx2.5 gene expression [29, 30] may also account for the absence of Nkx2.5 protein in cells positive for only ANF. Similarly, absence of ISL1 expression in E11.5 myocardial cells is in agreement with the reported downregulation of this gene during early cardiac development [8]. From these results we infer that undifferentiated myocardial cells lacking myofilaments and expressing only Nkx2.5 may represent putative CPCs while cells expressing Nkx2.5 and or ANF with apparent sarcomeric structures represent differentiating myocytes. We have recently used a real-time fluorescent reporter based approach to confirm that the E11.5 myocardial cells expressing only Nkx2.5 but not markers such as ANF and MLC-2V are indeed capable of differentiating into a more mature phenotype (McMullen *et al.*, in preparation).

Recent loss of function and gain of function studies have shed some light on the genes and factors responsible for heart development and morphogenesis [31]. However, more work needs to be done in this area to explain cardiac abnormalities and associated



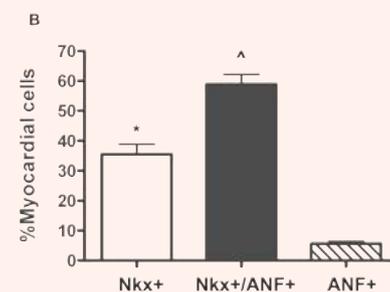
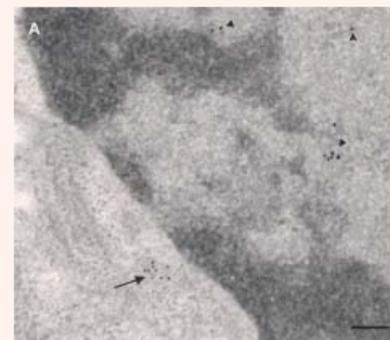
**Fig. 3** Immunogold labeling of undifferentiated myocardial cells. **(A)** A lower magnification of E11.5 undifferentiated cell processed for immunostaining with ANF antibodies, Scale bar = 500 nm. **(B)** A higher magnification of the boxed area in panel A showing the absence of immunogold probe directed against ANF, Scale bar = 100 nm. **(C)** Undifferentiated cell with clusters of immunogold probe directed against the transcription factor Nkx2.5 (arrowheads) in the nuclear compartment, Scale bar = 100 nm.



**Fig. 4.** Immunogold labeling of moderately differentiated and mature myocardial cells. (A and B) Clusters (arrowheads) of immunogold probe directed against the transcription factor Nkx 2.5 in a moderately differentiated cell (A) and a mature myocardial cell (B). Note the presence of well-organized sarcomeres (S) in the mature cell. Scale bars: A = 100 nm, B = 500 nm. (C and D) Clusters (arrowheads) of immunogold probe directed against ANF in a moderately differentiated cell (C) and a mature myocardial cell (D). Note the association of anti-ANF label with secretory granules. Scale bars: (C and D) = 100 nm.

embryonic deaths seen in several genetic models in which formation of a chambered heart is unperturbed or only modestly affected [32–37]. Our findings from this study suggest that multiple mechanisms may regulate further maturation of undifferentiated and moderately differentiated myocardial cells in later stages of cardiac development. We propose that any imbalances in regulatory mechanisms that can alter the ratio of undifferentiated cells to differentiated myocytes could result in late embryonic or postnatal mortalities reported for several genetic models [32–37]. For instance, retinoids were shown to play a key role in the early stages of cardiac development to prevent cell differentiation and favour cell proliferation [37]. Ablation of retinoid signaling pathway was shown to result in precocious myocardial cell maturation and early embryonic lethality [37]. Similarly, Nkx2.5 has been shown to regulate the transition between periods of cardiac induction, AHF progenitor cell proliferation, and outflow tract (OFT) morphogenesis *via* a Bmp2/Smad1 negative feedback loop [38]. Absence of such regulation in Nkx2.5 mutants was responsible for initial overspecification of progenitor cells followed by failed progenitor cell proliferation, OFT defects and early embryonic lethality [38].

In summary, our results suggest that myocardial cell differentiation is a gradual process in which a relatively small percentage of cells start to form organized sarcomeres at E11.5 and the proportion of differentiated cells increases dramatically in the later stages of



**Fig. 5** Double immunogold labeling of E11.5 ventricular myocardial cells. (A) Moderately differentiated myocardial cell with anti-Nkx2.5 label (10 nm gold particles, arrowheads) in the nuclear compartment and anti-ANF label (5 nm gold particles, arrow) in the cytoplasm, Scale bar = 100 nm. (B) Relative distribution of E11.5 ventricular myocardial cells positive for Nkx2.5 and/or ANF. Data is presented as mean value  $\pm$  S.E.M.,  $N = A$  total of 400 cells from three different hearts. \* $p < 0.005$  Nkx2.5<sup>+</sup> versus Nkx<sup>+</sup>/ANF<sup>+</sup> and ANF<sup>+</sup>,  $\wedge p < 0.005$  Nkx<sup>+</sup>/ANF<sup>+</sup> versus ANF<sup>+</sup>.

gestation. In this context, further work is needed to identify factors or mechanisms that are essential for proper maturation of undifferentiated cells in the embryonic heart post-chamber specification.

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