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Nestin expression is dynamically regulated in cardiomyocytes during embryogenesis

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Abstract

The transcriptional factors implicated in the expression of the intermediate filament protein nestin in cardiomyocytes during embryogenesis remain undefined. In the heart of 9,5–10,5 day embryonic mice, nestin staining was detected in atrial and ventricular cardiomyocytes and a subpopulation co-expressed Tbx5. At later stages of development, nestin immunoreactivity in cardiomyocytes gradually diminished and was absent in the heart of 17,5 day embryonic mice. In the heart of wild type 11,5 day embryonic mice, 54±7% of the trabeculae expressed nestin and the percentage was significantly increased in the hearts of Tbx5^{+/-} and Gata4^{+/-} embryos. The cell cycle protein Ki67 and transcriptional coactivator Yap-1 were still prevalent in the nucleus of nestin⁽⁺⁾-cardiomyocytes identified in the heart of Tbx5^{+/-} and Gata4^{+/-} embryonic mice. Phorbol 12,13-dibutyrate treatment of neonatal rat ventricular cardiomyocytes increased Yap-1 phosphorylation and co-administration of the p38 MAPK inhibitor SB203580 led to significant dephosphorylation. Antagonism of dephosphorylated Yap-1 signalling with verteporfin inhibited phorbol 12,13-dibutyrate/SB203580-mediated nestin expression and BrdU incorporation of neonatal cardiomyocytes. Nestin depletion with an AAV9 containing a shRNA directed against the intermediate filament protein significantly reduced the number of neonatal cardiomyocytes that re-entered the cell cycle. These findings demonstrate that Tbx5- and Gata4-dependent events negatively regulate nestin expression in cardiomyocytes during embryogenesis. By contrast, dephosphorylated Yap-1 acting via upregulation of the intermediate filament protein nestin plays a seminal role in the cell cycle re-entry of cardiomyocytes. Based on these data, an analogous role of Yap-1 may be prevalent in the heart of Tbx5^{+/-} and Gata4^{+/-} mice.

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Disclosures

None declared

Keywords

embryogenesis; cardiomyocytes; nestin; TBX5; GATA4; Yap-1

INTRODUCTION

Nestin is a member of the class VI family of intermediate filament proteins originally identified in neural progenitor/stem cells of the developing central nervous system. (Lendahl et al, 1990; Dahlstrand et al, 1992). However, additional studies revealed that nestin was not a unique marker of neural progenitor/stem cells as the intermediate filament protein was detected in fibroblasts (e.g. heart, kidney), vascular smooth muscle and endothelial cells, pre-existing cardiomyocytes bordering the peri-infarct region of the ischemically damaged human/rodent heart, skeletal muscle and tumorigenic cells (Sejerson and Lendahl, 1993; Carlsson et al, 1999; Vaitinen et al, 2001; Mokry et al, 2004; El-Helou et al, 2008; Oikawa et al, 2010; Tomita et al, 2010; Ishiwata et al, 2011; Beguin et al, 2012; Tardif et al, 2015; Hertig et al, 2017; Meus et al, 2017). Biologically, nestin plays a direct role in the proliferation and migration of normal and tumorigenic cells (Xue and Yuan, 2010; Ishiwata et al, 2011; Beguin et al, 2012; Liang et al 2015; Tardif et al, 2015; Matsuda et al 2016; Meus et al, 2017). Three specific regulatory intron regions identified in the nestin gene direct cell-specific expression of the intermediate filament protein. Intron 2 selectively directs nestin expression in neural progenitor/stem cells of the CNS and an analogous mechanism is prevalent in a resident population of neural progenitor/stem cells identified in the adult rodent heart (Zimmerman et al, 1994; Lothian et al, 1999; El-Helou et al, 2013). By contrast, intron 1 of the nestin gene drives expression of the intermediate filament protein in developing skeletal muscle, C2C12 myoblasts and endothelial cells and indirect data suggests a similar paradigm may be prevalent in the re-expression of nestin in pre-existing adult cardiomyocytes following myocardial infarction (Aihara et al, 2004; Zhong et al 2008; Meus et al, 2017). Kachinsky and colleagues were the first to identify nestin in cardiomyocytes during mid-embryonic development of the mouse heart and work from our lab reaffirmed the latter findings and further demonstrated that nestin depletion in rat embryonic cardiomyoblasts inhibited cell cycle re-entry (Kachinsky et al. 1995; Meus et al, 2017). Despite the established role of the various introns driving nestin expression in a cell-specific manner, the transcriptional factors directly involved in the induction and/or repression of the intermediate filament protein in embryonic cardiomyocytes remains presently unknown. The transcriptional factors Tbx5 (T-box transcription factor) and Gata4 (family of zinc finger transcription factors) play a seminal role in cardiomyocyte proliferation, chamber morphogenesis and specification during embryogenesis (Hatcher et al, 2001; Pu et al, 2004; Maitra et al, 2009; Misra et al, 2014; Ang et al, 2016). The HIPPO-regulated transcriptional coactivator Yes associated protein-1 (Yap-1) was reported to form a heterodimer with Tbx5 and may in part contribute to cardiac development initiated by Tbx5 during embryogenesis as depletion of Yap-1 compromised the proliferative response of embryonic cardiomyocytes leading to myocardial hypoplasia and lethality (Murakami et al, 2005; Xin et al, 2011). Based on their established proliferative roles, an important nexus may be prevalent coupling transcriptional factors Tbx5, Gata4 and/or Yap-1 to nestin expression during cardiogenesis. Employing transgenic and *in vitro* approaches, the present

study tested the hypothesis that Tbx5, Gata4 and Yap-1 regulate expression of the intermediate filament protein nestin in cardiomyocytes during embryonic development.

Materials and Methods

Ethics Approval

The use and care of laboratory rodents was according to the Canadian Council for Animal Care and approved by the Animal Care Committee of the Montreal Heart Institute.

Embryonic Mice

Embryonic day (E) 9,5 to 17,5 embryonic CD1 mice were used to assess the temporal pattern of nestin expression in cardiomyocytes (n=3–4 for each time point) (Charles Rivers, Canada). Cardiac specific Tbx5^{+/-} and Gata4^{+/-} E11,5 and E13,5 day mice were generated and genotyped, as previously described (Maitra et al, 2009; Misra et al, 2014). The mouse strain 129/C57BL6 was used to generate Gata4 heterozygous mice and Black Swiss/129 used to generate Tbx5 heterozygous mice. Wild type embryonic mice used were littermates of the Gata4 and Tbx5 heterozygotes. Briefly, as compared to wildtype mice, Tbx5^{+/-} and Gata4^{+/-} embryonic mice were characterized with a thin myocardium secondary to a reduced number of cardiomyocytes that expressed the G2-M marker phosphohistone-3 (Maitra et al, 2009; Misra et al, 2014).

Neonatal Rat Ventricular Cardiomyocytes

Neonatal rat ventricular cardiomyocytes were isolated from 1-day old Sprague-Dawley rat pups (sacrificed by decapitation) (Charles River, Canada) as previously described (Meus et al 2017). Ventricular cardiomyocytes were initially plated (400 cells/mm²) in DMEM-low glucose (Hyclone Laboratories, Logan, UT) supplemented with 7% heat-inactivated FBS and 1% penicillin-streptomycin for 24 hours, subsequently washed and maintained in DMEM-low glucose containing insulin (5 µg/ml), transferrin (5 µg/ml), and selenium (5 ng/ml) (ITS; BD Bioscience, Bedford, MA) for 24–36 hours prior to the experimental protocol.

Neonatal rat ventricular cardiomyocytes were co-treated for 24 hours with phorbol 12, 13-dibutyrate (10⁻⁷ M; Sigma-Aldrich, St. Louis, MO) and the p38 MAPK inhibitor SB203580 (10 µM; LC Laboratories, Woburn, MA) to induce the appearance of the intermediate filament protein nestin and initiate cell cycle re-entry as depicted by bromodeoxyuridine (BrdU; 20 µM; Sigma-Aldrich) incorporation added for a period of 24 hours. With regard to the 72 hour exposure, PDBu and SB were re-administered 24 and 48 hours after the initial treatment and BrdU was added during the last 24 hours of the experiment. In parallel experiments, verteporfin (100 and 250 nM; Sigma-Aldrich) and triciribine (5 µM; Selleckchem, Monmouth Junction, NJ) were added 15 minutes prior to the treatment with phorbol 12, 13-dibutyrate/SB203580. To assess Yap-1 phosphorylation, neonatal rat ventricular cardiomyocytes were treated with phorbol 12, 13-dibutyrate (10⁻⁷ M) in the absence or presence of SB203580 (10 µM). Neonatal ventricular cardiomyocyte hypertrophy was determined by ³H-leucine uptake. Experiments were performed on n=4–5 independent preparations of neonatal rat ventricular cardiomyocytes.

Immunofluorescence

Embryonic mice were immediately immersed in formalin and longitudinal sections of the whole animal or transverse sections of the thorax region were prepared, dehydrated and embedded in paraffin. Tissue sections of 6–8 μm thickness were deparaffinised, hydrated and treated to the antigen retrieval method (incubated in 10 mM Sodium Citrate (pH 6) at a temperature of 100°C for 30 minutes, allowed to return to ambient temperature and rinsed with PBS (pH 7.4). Primary antibodies include mouse monoclonal anti-nestin (1:200; Chemicon, Temecula, CA), chicken polyclonal anti-nestin (1:200; Abcam, Cambridge, MA) mouse monoclonal anti-cardiac troponin T (1:300; Abcam), rabbit polyclonal anti-Ki67 (1:200; Abcam), mouse monoclonal anti-Tbx5 (1:100; Santa Cruz Biotechnologies, Santa Cruz, CA), mouse monoclonal anti-Gata4 (1:100; Santa Cruz Biotechnologies) and rabbit polyclonal anti-Yap-1 (1:150; Cell Signalling, Danvers, MA). The nucleus was identified with TO-PRO@3 (1.5 μM ; emission wavelength, 661 nm; Life Technologies, Grand Island, NY) or 4',6'-diamidino-2-phenylindole (DAPI, 1.5 μM ; emission wavelength, 460 nm; Sigma-Aldrich) staining. Secondary antibodies employed were goat anti-mouse IgG conjugated to Alexa-555 or Alexa-647 (1:800; Life Technologies), goat anti-rabbit IgG conjugated to Alexa-488 or Alexa-647 (1:800; Life Technologies), goat anti-chicken IgG conjugated to Alexa-488 or Alexa-555 (1:800; Life Technologies), donkey anti-mouse IgG conjugated to Alexa-488 (1:800; Life Technologies), and a donkey anti-rabbit IgG conjugated to Alexa-555 (1:800; Life Technologies) (El-Helou et al. 2005, 2012). Non-specific staining was determined following the addition of the conjugated secondary antibody alone. To assess the percentage of trabeculae that expressed nestin in wildtype, Tbx5^{+/-} and Gata4^{+/-} E11.5 mice, three mice per group were used and at least 2–3 sections per mouse were examined. Regardless the level of staining, nestin immunoreactivity of trabeculae was considered a positive signal.

Primary passage neonatal rat ventricular cardiomyocytes were plated on coverslips coated with poly-D-lysine (0.1 mg/ml; Sigma-Aldrich) and following completion of the experimental protocol fixed with 2% paraformaldehyde. After fixation, ventricular cardiomyocytes were treated with 2 M HCL for 15 minutes to denature DNA. Thereafter, immunofluorescence was performed as previously described (Meus et al, 2017). Primary antibodies employed include mouse monoclonal anti-nestin (1:500), mouse monoclonal anti-cardiac troponin T (1:400; Abcam), rabbit polyclonal anti-collagen type I (1:400; Abcam) and chicken polyclonal anti-BrdU (1:500; Abcam). The nucleus was identified with TO-PRO@3 (Life Technologies) or 4',6'-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) staining. Secondary antibodies employed were goat anti-mouse IgG conjugated to Alexa-555 or Alexa-647 (1:800; Life Technologies), goat anti-rabbit IgG conjugated to Alexa-488 or Alexa-647 (1:800; Life Technologies), and a goat anti-chicken IgG conjugated to Alexa-488 or Alexa-555 (1:800; Life Technologies). Immunofluorescence of cells was visualized with a 10X- or 63X-oil 1.4 NA DIC plan apochromat objective mounted on a Zeiss Axiovert 100M confocal microscope.

Western Blot

Protein lysates of neonatal rat ventricular cardiomyocytes were prepared and subjected to SDS-electrophoresis, as previously described (Meus et al, 2017). Antibodies used include

rabbit polyclonal anti-Yap-1 (1:500; Cell Signalling), rabbit polyclonal directed against phosphorylated serine¹²⁷ of Yap-1 (1:500; Cell Signalling), and a mouse monoclonal anti-GAPDH (1:50,000; Ambion, Austin TX). Following overnight incubation at 4°C, the appropriate secondary antibody-conjugated to horseradish peroxidase (1:20,000, Jackson ImmunoResearch, West Grove, PA) was added and bands visualized utilizing the ECL detection kit (Perkin Elmer, Waltham, MA). Films were scanned with Image J software® and the target protein signal was depicted as arbitrary light units.

Short hairpin RNA (shRNA) Directed against Nestin and insertion in adeno-associated virus 9 (AAV9)

Short hairpin rat nestin with a mature antisense: 5'-TTCTAGATGGTTCACAATC targeting rat Nestin GB NM_012987 base positions 2833–2851 was generated by PCR and previously reported to effectively target the intermediate filament protein (Beguín et al, 2012; Tardif et al, 2015; Meus et al, 2017). Scrambled short hairpin sequence was taken from Addgene plasmid 1864. Briefly, single stranded cDNAs containing different short hairpin RNA flanked by HindIII and BamHI sites and 2 linkers were purchased and amplified by PCR using forward (5'-TAACCCTCACTAAAGGGACTC) and reverse (5'-TAATACGACTCACTATAGGGCTC) linker primers. Double stranded amplicons were digested with the 2 restriction sites, gel purified and ligated in pU6 ITR (donated by Dr. N. Boussette, Montreal Heart Institute) followed by transformation in Stbl3 bacteria. The clones were isolated and sequenced by Genome Quebec (Quebec, Canada).

Short hairpin targeting nestin;

5'TAACCCCTCACTAAAGGGACTCAAGCTTTTCCAAAAAAGATTGTGAACCATCTAGAACTCTTGATTCTAGATGGTTCACAATCCGGGATCCATCGAGCCCTATAGTGAGTCGTATTA

Scrambled short hairpin;

5'TAACCCCTCACTAAAGGGACTCAAGCTTTTCCAAAAAACCTAAGGTTAAGTCGCCCTCGCTCTTGACGAGGGCGACTTAACCTTAGGCGGGATCCATCGAGCCCTATAGTGAGTCGTATTA

Polyethylenimine (PEI-Max, Polysciences, Inc, Warrington, PA) was used to transfect HEK293T cells with the pDG9 and pU6 ITR containing the shRNA directed against nestin or the scrambled sequence. After 48 hours, cells were lysed and re-suspended in NaCl 5 M/ Tris 1M pH 8,5 and centrifuged. The pellet was re-suspended in lysis buffer, homogenized and exposed to 3 freeze (liquid nitrogen)/thaw (37°C) cycles. Thereafter, 1µl/ml of MgCl₂ was added and supplemented with 100 U/ml Benzonase (Sigma). The AAV9 was isolated using a discontinuous Iodixanol gradient and subsequently buffer exchanged to PBS using an Amicon Ultra filter 100,000 MWCO Centrifugation device (Millipore). The genomic titer of AAV9 virus containing the shRNA directed against nestin or scrambled sequence was determined by quantitative PCR. Neonatal rat ventricular cardiomyocytes in ITS serum-free media were infected with 10⁻⁷–10⁻⁸ viral particles for a period of 36–48 hours prior to experimental procedure.

Statistics

Data was presented as the mean $\pm$ S.E.M, (*n*) represents the number of mice and/or independent preparations of ventricular cells used per experiment. Data was evaluated by a student's unpaired t-test or a one-way ANOVA (Origin 2016, Northampton, MA) and a value of $P < 0.05$ was considered statistically significant.

RESULTS

Nestin immunoreactivity in the heart and skeletal muscle of embryonic mice

In the heart of E9,5 murine embryos, cytoplasmic staining of the type VI intermediate filament protein nestin was detected in cardiac troponin-T-immunoreactive atrial and ventricular cardiomyocytes (Figure 1A). Moreover, a partial striated phenotype of the intermediate filament protein nestin was apparent in cardiac troponin-T⁽⁺⁾-cardiomyocytes predominantly identified in the trabeculae of E10,5 and E11,5 mice (Figures 1B & 2A). Staining of the transcriptional factor Gata4 was observed in the heart of E10,5 mice and identified in the nucleus of nestin-immunoreactive cardiomyocytes (Suppl Figure 1). Nuclear staining of the transcriptional factor Tbx5 was also detected in a subpopulation of nestin⁽⁺⁾-cardiomyocytes in the heart of E10,5 mice (Suppl Figure 1). In the heart of E9,5–11,5 mice, nestin-immunoreactive epicardial and endothelial cells were also identified (Figures 1 & 2) and further characterized by nuclear Gata4 staining (Suppl Figure 1). The latter data was consistent with previous studies highlighting the presence of embryonic endothelial and epicardial cells that expressed nestin and the transcriptional factor Gata4 (Watt et al, 2004; Wagner et al, 2006). At E13,5 (Suppl Figure 2), residual nestin staining was observed in embryonic cardiomyocytes identified in the compact myocardium and trabeculae whereas immunoreactivity of the intermediate filament protein was undetectable in cardiac troponin-T⁽⁺⁾-atrial and ventricular cardiomyocytes of E17,5 hearts (Figure 1). However, nestin-immunoreactive endocardial and epicardial cells persisted in E13,5 (Suppl Figure 2) and E17,5 hearts (Figure 1). Despite the temporal reduction of nestin staining of cardiomyocytes in the developing embryonic heart, the intermediate filament protein and cardiac troponin-T were robustly co-expressed in skeletal muscle of E13,5 mice (Suppl Figure 3) and the phenotype persisted at E17,5 (data not shown). Nestin immunoreactivity was also identified in the developing spinal cord and lungs of E13,5 (Suppl Figure 3) and E17,5 (data not shown) mice.

The percentage of nestin-immunoreactive trabeculae was increased in the heart of Tbx5^{+/-} and Gata4^{+/-} heterozygous mice

A semi-quantitative analysis revealed that 54 $\pm$ 7% of the trabeculae identified in the heart of E11,5 mice (*n*=3) expressed the intermediate filament protein nestin and was partially organized in a striated pattern (Figures 2A, 2D, 3A). In the heart of E11,5 Tbx5^{+/-} mice (*n*=3), nestin staining of trabeculae was apparently greater, uniform and partially striated and the percentage of trabeculae expressing the intermediate filament protein was significantly higher as compared to the heart of wildtype embryonic mice (Figures 2B, 2D & 3B). However, in contrast to wildtype mice, the physical interaction between nestin-immunoreactive endocardial cells and developing trabeculae was disrupted in the heart of Tbx5^{+/-} embryos. Analogous to Tbx5 heterozygous mice, nestin staining of trabeculae in the

heart of $Gata4^{+/-}$ E11,5 mice ($n=3$) was also observed in a partial striated pattern and expression was apparently greater versus wild type mice and reaffirmed by the significant increased percentage of trabeculae immunoreactive to the intermediate filament protein (Figures 2C, 2D & 3C). The increased appearance of the intermediate filament nestin in the trabeculae of the heart of $Tbx5^{+/-}$ and $Gata4^{+/-}$ embryonic mice persisted at E13,5 (Suppl Figure 2). It has been reported that cardiomyocyte proliferation characterized by the appearance of phosphohistone-3 (e.g. marker of G2-M phase) was partially compromised in $Tbx5^{+/-}$ and $Gata4^{+/-}$ embryonic mice and translated to reduced ventricular wall thickness (Maitra et al, 2009; Misra et al, 2014). In wild type mice, Ki67 nuclear staining was identified in embryonic cardiomyocytes expressing the intermediate filament protein nestin (Figure 2A). An analogous paradigm was observed in $Tbx5^{+/-}$ and $Gata4^{+/-}$ mice revealing that despite a compromised transition to G2-M, cell cycle re-entry into the G1-S phase persisted in nestin-immunoreactive embryonic cardiomyocytes (Figures 2B & 2C).

The relationship between the HIPPO signaling pathway, nestin expression and cell cycle re-entry in embryonic and neonatal ventricular cardiomyocytes

Dephosphorylated Yap-1 translocates to the nucleus and interacts with the transcriptional factor TEAD (TEA domain family) leading to the expression of a panel of genes implicated in cellular proliferation (Johnson and Halder, 2014). In wildtype E11,5 mice, the transcriptional coactivator Yap-1 was identified in the nucleus of cardiomyocytes expressing the intermediate filament protein nestin (Figure 3A). Nuclear Yap-1 staining was also detected in epicardial and endocardial nestin⁽⁺⁾-cells (Figures 3A, 3B & 3C). A similar phenotype was recently reported as Yap-1 was detected in endocardial cells of the embryonic heart and was instrumental in driving endothelial mesenchymal transition and subsequent heart valve development (Zhang et al, 2014). In $Tbx5^{+/-}$ and $Gata4^{+/-}$ mice E11,5 mice, nuclear Yap-1 staining persisted in nestin⁽⁺⁾-cardiomyocytes indirectly suggesting that persistent expression of the transcriptional coactivator in the presence of reduced $Tbx5$ and $Gata4$ levels may have contributed in part to the increased percentage of trabeculae expressing the intermediate filament protein (Figures 3B & 3C). To investigate the latter premise, the relationship between Yap-1, nestin expression and cell cycle re-entry was examined in neonatal rat ventricular cardiomyocytes. Work from our lab has previously reported that treatment of 1-day old neonatal rat ventricular cardiomyocytes with the phorbol ester phorbol 12,13-dibutyrate (100 nM) increased p38 MAPK phosphorylation and the p38 MAPK kinase inhibitor SB203580 (10 μ M) completely inhibited kinase activity as reflected by the abrogation of HSP27 phosphorylation (Meus et al, 2017). The co-treatment of neonatal rat ventricular cardiomyocytes with phorbol 12,13-dibutyrate/SB203580 led to the upregulation of the intermediate filament protein nestin and concomitant cell cycle re-entry (Meus et al, 2017). In the present study, the exposure of neonatal rat ventricular cardiomyocytes with phorbol 12,13-dibutyrate (100 nM) alone failed to induce nestin expression and cell cycle re-entry (data not shown) but led to a significant hypertrophic response as reflected by ³H-leucine uptake ($86 \pm 16\% \uparrow$ versus untreated; $n=4$). The 24 hour co-treatment with phorbol 12,13-dibutyrate (100 nM) and SB203580 (10 μ M) increased the number of neonatal rat ventricular cardiomyocytes that expressed nestin and re-entered the cell cycle as depicted by the incorporation of the nucleotide analog bromodeoxyuridine (BrdU) (Figures 4A & 5A). However, the hypertrophic response of neonatal rat ventricular

cardiomyocytes following a 24 hour exposure to phorbol 12,13-dibutyrate persisted after the co-treatment with SB203580 ($73 \pm 7\% \uparrow$ versus PDBu alone). Following a 72 hour co-treatment with phorbol 12,13-dibutyrate/SB203580, ~50% of neonatal rat ventricular cardiomyocytes expressed nestin and ~40% of the latter population had re-entered the cell cycle (Figures 4B & 5A). The acute treatment of neonatal rat ventricular cardiomyocytes with phorbol 12,13-dibutyrate alone (100nM) robustly promoted the phosphorylation of the serine¹²⁷ residue of the transcriptional coactivator Yap-1 (Figures 6A & 6B). The co-administration of SB203580 significantly attenuated phorbol 12,13-dibutyrate mediated phosphorylation of the serine¹²⁷ residue of Yap-1 albeit complete dephosphorylation was not observed (Figures 6A & 6B). To elucidate the role of dephosphorylated Yap-1, the pharmacological agent verteporfin which inhibits the interaction between Yap-1 and TEAD transcriptional factors was employed (Wei et al, 2016). Verteporfin administration (100 and 250 nM) led to a dose-dependent suppression of nestin expression and Brdu incorporation by neonatal rat ventricular cardiomyocytes when added prior to phorbol 12,13-dibutyrate/SB203580 (Figures 4D & 5B).

To determine the biological role of nestin in cell cycle re-entry, the intermediate filament protein was selectively targeted via a shRNA approach. AAV9 infection of neonatal rat ventricular cardiomyocytes containing the shRNA directed against nestin significantly inhibited phorbol 12,13-dibutyrate/SB203580-mediated expression of the intermediate filament protein and concomitantly attenuated cell cycle re-entry as compared to cardiomyocytes infected with the scrambled shRNA (Figure 5C). The phosphoinositide-3 kinase/phosphoinositide-dependent protein kinase/protein kinase B (PI3-K/PDK/AKT) signalling pathway was reported to mediate the proliferative response facilitated by the intermediate filament protein nestin (Xue and Yuan, 2010; XB Zhen et al, 2014; Liu et al 2015). To examine the relationship between nestin and PI3-K/PDK/AKT, neonatal rat ventricular cardiomyocytes were treated with the molecule triciribine that inhibits PDK1 phosphorylation of the threonine³⁰⁸ residue of AKT (Berndt et al, 2010). Triciribine (5 μ M) addition to phorbol 12,13-dibutyrate/SB203580-treated neonatal rat ventricular cardiomyocytes significantly increased the number of cardiomyocytes that expressed nestin but concomitantly inhibited BrdU incorporation (Figures 4C & 5D).

DISCUSSION

During the early stages of embryonic heart development, the splanchnic and pharyngeal mesoderm give rise to cardiac precursor cells of the first and second heart field, respectively and collectively interact in cardiac morphogenesis and differentiation (Paige et al, 2015). The first heart field cells are identified by the transcriptional factor Tbx5 and contribute significantly to the formation of the left ventricle and have a modest role in atrial formation (Paige et al, 2015). By contrast, cardiomyocytes derived from the second heart field are characterized by the expression of the LIM homeodomain transcriptional factor Islet 1 (ISL1) and play a major role in the formation of the right ventricle, outflow tract and atria (Paige et al, 2015). In the present study, nestin immunoreactivity was detected in embryonic cardiomyocytes in the heart of E9.5–11.5 mice and a subpopulation co-expressed Tbx5 suggesting that the intermediate filament protein may represent an additional phenotype of cardiac precursor cells derived from the first heart field. Nestin immunoreactivity was still

detectable in the compact myocardium and a subpopulation of cardiomyocytes identified in the trabeculae of E13,5 murine embryos whereas staining was absent in cardiomyocytes of E17,5 mice. In the adult rodent heart, nestin was undetectable in ventricular cardiomyocytes whereas re-expression of the intermediate filament protein in a partially striated pattern was observed in pre-existing ventricular cardiomyocytes bordering the peri-infarct region and an analogous paradigm was observed in the ischemically damaged human heart (El-Helou et al, 2008; Mokry et al, 2008; Meus et al, 2017). These data support the premise that re-expression of nestin in pre-existing cardiomyocytes of the ischemically damaged adult mammalian heart recapitulated an embryonic phenotype. Moreover, despite the progressive temporal loss of nestin in the developing embryonic heart, the intermediate filament protein and cardiac troponin-T were highly expressed in skeletal muscle of E13,5 and E17,5 mice. The latter findings were consistent with previous studies identifying nestin and cardiac troponin-T expression in developing skeletal muscle (Sejerson and Lendahl, 1993). During postnatal development, nestin and cardiac troponin-T expression gradually diminishes but residual nestin staining was still detected at the myotendinous and neuromuscular junctions of adult skeletal muscle (Sejerson and Lendahl, 1993; Carlsson et al, 1999). Following injury, nestin and cardiac troponin-T were re-expressed in skeletal muscle and akin to that observed in the ischemically damaged heart, recapitulated an embryonic phenotype (Vaittinen et al, 2001).

Cardiogenesis is a complex process tightly regulated by transcriptional factors Gata4 and Tbx5 that act in a coordinated fashion to initiate cardiomyocyte proliferation, chamber morphogenesis and specification (Hatcher et al, 2001; Pu et al, 2004; Maitra et al, 2009; Misra et al, 2014; Ang et al, 2016). Consistent with this premise, mutations of Gata4 or Tbx5 lead to cardiac birth defects and congenital heart disease (Paige et al, 2015). The established proliferative role of Gata4 and Tbx5 on embryonic cardiomyocytes provided the impetus to assess whether the latter paradigm was mediated in part via upregulation of the intermediate filament protein nestin. As previously reported, nestin was directly responsible for the cell cycle re-entry of embryonic rat cardiomyoblasts (H9c2 cells) and the intermediate filament protein plays a seminal role in the proliferative response of normal and tumorigenic cells (Xue and Yuan, 2010; Ishiwata et al, 2011; Beguin et al, 2012; Liang et al 2015; Tardif et al, 2015; Matsuda et al 2016; Meus et al, 2017). Previous studies have reported that myocardial thinning was evident in the atria and ventricle of E11,5 to E13,5 Tbx5^{+/-} and Gata4^{+/-} embryonic mice attributed in part to a significant decrease in the number of cardiomyocytes that entered the G2-M phase, as depicted by phosphohistone-3 immunoreactivity (Maitra et al, 2009; Misra et al, 2014). However, in contrast to a postulated downregulation of nestin in the heart of E11,5 Tbx5^{+/-} and Gata4^{+/-} embryonic mice, the percentage of trabeculae that expressed the intermediate filament protein was significantly increased as compared to wildtype mice and the response persisted in the heart of heterozygous E13,5 embryonic mice. These data support the novel premise that Gata4- and Tbx5-dependent events negatively regulate nestin expression during embryonic development of the heart. Moreover, nuclear Ki67 staining persisted in nestin⁽⁺⁾-cardiomyocytes identified in the trabeculae of Tbx5^{+/-} and Gata4^{+/-} embryonic mice demonstrating that despite a compromised proliferative response, cell cycle re-entry was still prevalent. Indeed, in mice overexpressing the human nestin gene, the number of embryonic

cardiomyocytes that re-entered the cell cycle as determined by BrdU incorporation was significantly increased (Liu et al, 2015). Thus, at least in the heart of $Tbx5^{+/-}$ and $Gata4^{+/-}$ embryonic mice, nestin upregulation alone in cardiomyocytes was apparently insufficient to progress from G1-S to G2-M.

HIPPO-regulated transcriptional coactivators Yap-1 and TAZ were reported to associate with $Tbx5$ and influence $Tbx5$ -dependent gene expression during embryogenesis (Murakami et al, 2005). Akin to $Tbx5$ and $Gata4$, dephosphorylated Yap-1 directly promotes embryonic cardiomyocyte proliferation leading to a thickened compact myocardium and expanded trabecular layer (Xin et al, 2011). Furthermore, dysregulation of the HIPPO pathway in the adult rodent heart led to the nuclear accumulation of dephosphorylated Yap-1 initiating cell cycle re-entry of cardiomyocytes and cardiac regeneration following an ischemic insult (Heallen et al, 2013). Based on these observations, the reduced expression of the transcriptional factor $Tbx5$ and possibly $Gata4$ in heterozygous embryonic mice may re-orient the biological targets of the transcriptional coactivator Yap-1 during cardiac embryogenesis (Ang et al, 2016). Moreover, it has been reported that acinar ductal metaplasia was characterized by the upregulation of nestin and progression of the disease involved the nuclear accumulation of Yap-1/TAZ (Means et al, 2005; Gruber et al, 2016). These observations collectively provided the impetus to examine whether Yap-1 signaling in $Tbx5^{+/-}$ and $Gata4^{+/-}$ embryonic mice could have contributed in part to the increased percentage of trabeculae that expressed nestin. Yap-1 immunoreactivity was detected in the nucleus of nestin⁽⁺⁾-cardiomyocytes of wildtype, $Tbx5^{+/-}$ and $Gata4^{+/-}$ mice suggesting that the transcriptional coactivator was biologically active. To directly examine the relationship between Yap-1 and nestin, neonatal rat ventricular cardiomyocytes were used as a surrogate model. Work from our lab has previously reported that the serine/threonine kinase p38 MAPK suppressed nestin expression and cell cycle re-entry of neonatal rat ventricular cardiomyocytes in response to the protein kinase C activator phorbol 12,13-dibutyrate (Meus et al, 2017). The HIPPO signalling pathway was identified as a target of p38 MAPK as oxidative stress-mediated recruitment of the serine/threonine kinase led to Yap-1 phosphorylation/inactivation and cytoplasmic retention (Lee and Yonehara, 2012). However, Yap-1 was not identified as a direct target of p38 MAPK thereby suggesting that phosphorylation of the transcriptional coactivator occurred via p38 MAPK-mediated phosphorylation/recruitment of Mst1/2 and/or Lats1/2 (Lee and Yonehara, 2012). The hypertrophic response of neonatal rat ventricular cardiomyocytes in response to phorbol 12,13-dibutyrate was associated with increased phosphorylation of the serine¹²⁷ residue of Yap-1. This observation was consistent with a previous study reporting phorbol ester mediated Yap-1 phosphorylation and inactivation in Swiss 3T3, MEF, and lung cancer A549 cells (Gong et al, 2015). Thus, at least in response to phorbol 12,13-dibutyrate, Yap-1 was dispensable for the ensuing hypertrophic response of neonatal cardiomyocytes. However, addition of the p38 MAPK inhibitor SB203580 to phorbol 12,13-dibutyrate stimulated neonatal rat ventricular cardiomyocytes led to Yap-1 dephosphorylation, albeit residual phosphorylation of the transcriptional coactivator persisted. Antagonism of dephosphorylated Yap-1 signaling following the addition of verteporfin to phorbol 12,13-dibutyrate/SB203580-treated neonatal rat ventricular cardiomyocytes led to a significant dose dependent inhibition of nestin expression and cell cycle re-entry. Collectively, these

data reveal that p38 MAPK directly influenced the HIPPO signalling pathway and Yap-1 plays a seminal role in nestin expression and cell cycle re-entry of neonatal rat ventricular cardiomyocytes. In this regard, the persistent expression of nuclear Yap-1 in cardiomyocytes in the heart of *Tbx5*^{+/-} and *Gata4*^{+/-} embryonic mice may have contributed in part to the increased percentage of nestin⁽⁺⁾-trabeculae. Lastly, the relationship between Yap-1 and nestin may not be exclusive to cardiomyocytes as dysregulation of the HIPPO pathway and the intermediate filament protein were both linked to the development of various forms of cancer (Ishiwata et al, 2011; Johnson and Halder, 2014).

Despite establishing a role of Yap-1 in nestin expression, the biological impact of the intermediate filament protein on cell cycle re-entry of neonatal rat ventricular cardiomyocytes remains unknown. To address this issue, neonatal rat ventricular cardiomyocytes were infected with the AAV9 containing a scrambled shRNA or shRNA directed against nestin. Previous work from our lab has shown that the shRNA employed in the present study successfully depleted nestin expression in neonatal rat ventricular fibroblasts, rat vascular smooth muscle cells and H9c2 cells (Beguín et al, 2012; Tardif et al, 2015; Meus et al 2017). Treatment with the AAV9 containing the shRNA directed against nestin significantly reduced the number of neonatal rat ventricular cardiomyocytes that expressed the intermediate filament protein and re-entered the cell cycle. The data establish a direct role of nestin in the cell cycle re-entry of neonatal rat ventricular cardiomyocytes and reaffirms the proliferative role of the intermediate filament protein previously reported in normal and tumorigenic cells. Moreover, these findings were consistent with the observation that nestin⁽⁺⁾-cardiomyocytes identified in wildtype, *Tbx5*^{+/-} and *Gata4*^{+/-} embryonic mice had entered the cell cycle as depicted by the nuclear expression of the cell cycle protein Ki67.

In tumorigenic and neural progenitor/stem cells as well as the embryonic mouse heart, cell cycle re-entry facilitated by the intermediate filament protein nestin was associated with the recruitment of the signalling axis PI3K/PDK/AKT (Xue and Yuan, 2010; XB Zhen et al, 2014; Liu et al 2015). Moreover, PI3K/PDK/AKT signalling was reported to promote the cell cycle re-entry of embryonic and adult cardiomyocytes (Evans-Anderson et al, 2008; Beigi et al, 2013; Lin et al, 2015). Furthermore, a recent study by Lin and colleagues revealed that Yap-1 mediated cardiomyocyte proliferation and survival required PI3K/PDK/AKT signaling (Lin et al, 2015). These data and the findings obtained in the present study suggest that Yap-1 mediated recruitment of PI3K/PDK1/AKT may have occurred in part via upregulation the intermediate filament protein nestin. To examine the latter premise, neonatal rat ventricular cardiomyocytes were treated with the pharmacological agent triciribine reported to inhibit AKT phosphorylation by binding to the pleckstrin homology domain and preventing association with PDK (Berndt et al, 2010). The co-administration of triciribine to phorbol 12,13-dibutyrate/SB203580-treated neonatal rat ventricular cardiomyocytes significantly inhibited BrdU incorporation by nestin⁽⁺⁾-cardiomyocytes but concomitantly increased the number of cardiomyocytes that expressed the intermediate filament protein. Thus, the pharmacological data suggest that nestin upregulation via a Yap-1-dependent pathway facilitated the cell cycle re-entry of neonatal rat ventricular cardiomyocytes by recruiting the PI3K/PDK/AKT signaling axis. However, the significant increase in the number of nestin⁽⁺⁾-neonatal rat ventricular cardiomyocytes

following triciribine administration suggests that Yap-1 mediated proliferation via PI3K/PDK/AKT was sensitive to negative feedback regulation by AKT. Indeed, at least two studies have reported that the serine¹²⁷ residue of Yap-1 was phosphorylated by the serine/threonine kinase AKT leading to cytoplasmic retention and elimination of its transcriptional role as a coactivator (Basu et al, 2003; Zhang et al, 2012).

The present study has revealed that the intermediate filament protein nestin was expressed during the early phase of embryonic cardiac development and detected in a subpopulation of Tbx5-immunoreactive cardiomyocytes. The transcriptional factors Tbx5 and Gata4 negatively regulate nestin expression in embryonic cardiomyocytes whereas the coactivator Yap-1 may play a seminal role in the induction of the intermediate filament protein. Employing an adenovirus-shRNA approach, nestin depletion significantly reduced the number of neonatal rat ventricular cardiomyocytes that incorporated BrdU reaffirming the previous described role of the intermediate filament protein in the cell cycle re-entry of normal and tumorigenic cells. Moreover, nestin-mediated BrdU incorporation by neonatal rat ventricular cardiomyocytes required the recruitment of the PI3K/PDK/AKT signaling axis. These data support the premise that nestin expression in embryonic cardiomyocytes contributes in part to cell cycle re-entry but may not be sufficient to promote the progression from G1-S to G2-M in Tbx5^{+/-} and Gata4^{+/-} embryonic mice. Lastly, it remains presently unknown if the increased expression of the intermediate filament protein nestin may significantly alter the phenotype of embryonic cardiomyocytes and subsequently contribute in part to the reported cardiac birth defects and congenital heart disease associated with reduced expression of Tbx5 and Gata4.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A) Ventricle E9,5 days **B) Atria E9,5 days**

Figure 1. Nestin immunoreactivity of embryonic cardiomyocytes

(Panels A & B) In the heart of E9,5 mice, nestin immunoreactivity (red fluorescence) was detected in cardiac troponin-T⁽⁺⁾- (green fluorescence) atrial and ventricular cardiomyocytes. (Panel C) In the heart of E10,5 mice, nestin immunoreactivity persisted in cardiac troponin-T⁽⁺⁾-cardiomyocytes and was further organized in a partial striated phenotype. (Panel D) In the heart of E17,5 mice, nestin immunoreactivity in cardiomyocytes was absent. Regardless the time point, endocardial and epicardial cells identified in the

embryonic heart expressed the intermediate filament protein nestin (indicated by arrow). Nuclear staining was identified with TO-PRO®3 or 4',6'-diamidino-2-phenylindole (blue fluorescence).

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A) WT E11,5 days **B) TBX5^{+/-} e11,5 days**

Figure 2. Nestin immunoreactivity in the heart of E11,5 Tbx5^{+/-} and Gata4^{+/-} embryonic mice (Panel A) In the heart of E11,5 wildtype mice, a subpopulation of cardiac troponin-T⁽⁺⁾-cardiomyocytes (green fluorescence) identified in trabeculae were characterized by nestin immunoreactivity (red fluorescence) and associated with the nuclear staining of the cell cycle protein Ki67 (white fluorescence; indicated by arrow). **(Panels B & C)** In the heart of E11,5 Tbx5^{+/-} and Gata4^{+/-} mice, nestin staining of cardiac troponin-T⁽⁺⁾-cardiomyocytes identified in trabeculae was partially striated and apparently greater as compared to the heart of wildtype embryonic mice. Furthermore, nuclear Ki67 staining persisted in nestin⁽⁺⁾-cardiomyocytes (indicated by arrow). Nestin immunoreactivity was also detected in endocardial and epicardial cells (indicated by arrowhead) in the heart of wildtype, Tbx5^{+/-} and Gata4^{+/-} mice. **(Panel D)** A semi-quantitative assessment revealed that the percentage

of trabeculae that expressed the intermediate filament protein nestin in the heart of $Tbx5^{+/-}$ and $Gata4^{+/-}$ mice was significantly greater than wildtype mice. Experiments were performed on $n=3$ mice per group and (*) denotes $P<0.05$ versus wildtype.

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A) WT e11,5 days **B) TBX5 Het e11,5 days**

C) GATA4 Het e11,5 days **Figure 3. Nuclear Yap-1 staining of nestin⁽⁺⁾-embryonic cardiomyocytes**

(Panel A) In the heart of E11,5 wildtype mice, a subpopulation of cardiac troponin-T⁽⁺⁾-cardiomyocytes identified in trabeculae (green fluorescence) were characterized by nestin immunoreactivity (red fluorescence) and associated with the nuclear staining of the transcriptional coactivator Yap-1 (white fluorescence; indicated by arrow). **(Panels B & C)** In the heart of E11,5 Tbx5^{+/-} and Gata4^{+/-} mice, nestin staining of trabeculae was partially striated and greater and nuclear Yap-1 staining persisted (indicated by arrow). Yap-1 immunoreactivity was also detected in endocardial and epicardial cells (indicated by arrowhead) in the heart of wildtype, Tbx5^{+/-} and Gata4^{+/-} mice. Nuclear staining was identified with TO-PRO®3 or 4',6'-diamidino-2-phenylindole (blue fluorescence).

Figure 4. Dynamic regulation of nestin expression in neonatal rat ventricular cardiomyocytes (**Panel A**) A 24 hour exposure to phorbol 12,13-dibutyrate (PDBu; 10^{-7} M) and the p38 MAPK inhibitor SB203580 (SB; $10\mu\text{M}$) induced the appearance of the intermediate filament protein nestin (red fluorescence) and incorporation of the nucleotide analog BrdU (white fluorescence; indicated by arrow) in cardiomyocytes characterized by the absence of collagen type I staining (green fluorescence). (**Panel B**) The response to PDBu/SB persisted following a 72 hour exposure and a greater number of ventricular cardiomyocytes expressed nestin and incorporated BrdU (indicated by arrow). (**Panel C**) The co-administration of triciribine ($5\mu\text{M}$) increased the number of ventricular cardiomyocytes that expressed nestin in response to PDBu/SB but concomitantly inhibited BrdU incorporation (indicated by arrow). (**Panel D**) The co-administration of verteporfin (VER; 250 nM) reduced the expression of nestin and BrdU incorporation by ventricular cardiomyocytes in response to PDBu/SB. Nuclear staining was identified with TO-PRO@3 or 4',6'-diamidino-2-phenylindole (blue fluorescence).

Figure 5. Semi-quantitative analysis of nestin expression and BrdU incorporation by neonatal rat ventricular cardiomyocytes

(Panel A) A 24 and 72 hour exposure to phorbol 12,13-dibutyrate (PDBu; 10^{-7} M) and the p38 MAPK inhibitor SB203580 (SB; $10\mu\text{M}$) significantly increased the number of neonatal ventricular cardiomyocytes that expressed nestin and incorporated the nucleotide analog BrdU. (*) denotes $P < 0.05$ versus 24 hr treatment. **(Panel B)** The co-administration of verteporfin (VER) led to a dose-dependent inhibition of the number of neonatal ventricular cardiomyocytes that expressed nestin and incorporated BrdU following a 24 hour exposure to PDBu/SB. (*) denotes $P < 0.05$ versus PDBu/SB treatment. **(Panel C)** The infection of neonatal ventricular cardiomyocytes with an AAV9 containing a shRNA directed against nestin significantly reduced the number that expressed the intermediate filament protein and incorporated BrdU, as compared to cardiomyocytes infected with the AAV9 containing a scrambled shRNA. (*) denotes $P < 0.05$ versus scrambled and $n=7$ per group. **(Panel D)** The co-administration of tricyribine (TRI) significantly increased the number of ventricular cardiomyocytes that expressed nestin but inhibited the incorporation of BrdU following a 24 hour exposure to PDBu/SB. (*) denotes $P < 0.05$ versus PDBu/SB treatment.

Figure 6. The p38 MAPK inhibitor SB203580 inhibits phorbol ester-mediated YAP-1 phosphorylation

(Panels A & B) The acute exposure of neonatal rat ventricular cardiomyocytes to phorbol 12,13-dibutyrate (PDBu; 10⁻⁷ M) increased the phosphorylation of the serine¹²⁷ residue of Yap-1. The co-treatment with the p38 MAPK inhibitor SB203580 (SB; 10 μM) significantly attenuated PDBu-mediated Yap-1 phosphorylation. (*) denotes $P < 0.05$ between PDBu and PDBu/SB treatment.