Divergent cell cycle kinetics of midgestation ventricular cells entail a higher engraftment efficiency after cell transplantation

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LINEAGE TRACING EXPERIMENTS have indicated that the mammalian heart is derived from two separate heart-forming fields in the developing embryo. Cardiac progenitor cells (CPCs) present in the classical primary heart field (PHF) were shown to contribute to all major cell types in the heart. In contrast, CPCs present in the anterior heart field (AHF) were shown to contribute mainly to the formation of right ventricle and outflow tract (OFT) (2). The transcription factor Nkx2.5 is the earliest known marker expressed in both heart fields while the LIM/homeodomain gene Islet 1 (Isl1) is only expressed in AHF descendants (2, 3, 15, 31). In response to factors secreted from the adjacent endoderm, heart field progenitors proliferate extensively and differentiate into committed cell lineages (33). In later stages of development, the primitive heart is transformed into a fully functional structure by a variety of morphogenetic events such as chamber morphogenesis, septation, valve formation, and vasculogenesis (4, 33, 36). Stage-specific regulation of these events by a variety of growth factors secreted by the endocardium, epicardium, myocardium, atrioventricular cushion tissue, and resident fibroblasts during subsequent developmental stages clearly underscores the complexity of cardiac morphogenesis (20, 22, 38).

Recent studies have identified rare populations of myogenic stem/progenitor cells in the neonatal and adult hearts which appear to be distinct from each other in terms of marker gene expression (1, 15, 16, 21). These findings raise the possibility that some CPCs may remain undifferentiated in the embryonic heart even after chamber specification. Indeed, additional studies have confirmed the presence of Nkx2.5 or Isl1 positive and sarcomere negative CPCs in the myocardial layer interspersed with sarcomere-containing cells (15, 17, 39). While it is well known that the cells in the primitive heart tube retain high proliferation rates compared with myocytes in the fully formed embryonic heart (8, 26), there is no information on the cell cycle profiles and the differentiation potential of residual CPCs present in the midgestation ventricles (17, 39). Similarly, the cell cycle dynamics of the primary or secondary heart field lineages have not been well established. The discovery of functional coupling between transplanted embryonic cardiomyocytes and host myocardial cells (25) and the ability of transplanted cardiomyocytes to confer protection against the induction of arrhythmia (24, 29) are significant steps toward the use of cell-based therapies for myocardial repair. However, it is not known whether the developmental stage and/or differentiation status of the embryonic donor cells would have a direct impact on the graft size, which remains a major challenge in the donor cell transplantation field (18).

The present study was conducted to address mainly two original objectives using innovative lineage tracking methodologies. The first objective was to examine the cell cycle and differentiation dynamics of residual cardiac progenitor cells (CPCs) present in mid- (E11.5) and late- (E14.5) gestation embryonic ventricles and the second objective was to examine whether the developmental stage of embryonic ventricular cells would have any impact on graft size after cell transplantation. Here, we show that although the number of residual CPCs significantly decreases between mid- and late-gestation ventricular development, the cell cycle activity of these residual CPCs remains significantly higher (2-fold) in E11.5 ventricles compared with E14.5 ventricles, and the E11.5 CPCs differentiate into functional cardiomyocytes. Moreover, we also...
show that higher cell cycle activity of both CPCs and cardiomyocytes at midgestational stage enables E11.5 cells to form larger grafts compared with cells derived from E14.5 ventricles. To the best of our knowledge, this is the first report which shows that the developmental staging of ventricular cells might be useful for maximizing the beneficial effects of donor cell transplantation in myocardial repair.

MATERIALS AND METHODS

Experimental animals and genotyping. All animal procedures were performed according to the Canadian Council on Animal Care guidelines and were approved by the University Committee on Laboratory Animal Care (Protocol 10-008; 09-038, 12-013). Generation of mice with Cre recombinase inserted into Nkx2.5 (34) or Isl1 (32) alleles was previously described. The R26R-lacZ or EGFP reporter strains (ROSA-lacZ or ROSA-EGFP) were obtained from the Jackson Laboratory (Bar Harbor, ME). All transgenic lines were maintained in C57BL/6J (BL6) background. For genotyping, genomic DNA was extracted from ear punch biopsies and a PCR amplification assay was performed using RedExtract amplification kit (Sigma) and appropriate primer sets for each transgenic line (Table 1). For control comparisons, BL6 and CD1 mice were purchased from Jackson Laboratory and Charles River Laboratory (Wilmington, MA), respectively. Female mice were mated with males, and noon time on the day when the copulation plug was found was designated as embryonic (E) day 0.5 (E0.5).

Lineage tracking in whole mount embryos or dispersed cell preparations and [3H]thymidine labeling assay. Whole embryos and embryonic hearts from different developmental stages were dissected under a stereomicroscope (MZ16F, Leica). For dispersed ventricular cell preparations, tissue samples were digested in 0.2% type I Collagenase (Worthington, NJ), and cells were cultured on fibronectin-coated chamber slides (Nalge Nunc, NY) in 10% FBS-DMEM for 10 h. Subsequently, cells were pulsed with [3H]thymidine (GE Healthcare, 1.0 μCi/ml of medium) for 6 h, fixed with Flow fix (2.7% wt/vol cacodylic acid, 2.5% wt/vol paraformaldehyde, 1.66% wt/vol NaCl in ddH2O, pH 7.4) for 30 min at 4°C, processed for X-Gal staining and in situ [3H]thymidine autoradiography as described earlier (23). For whole mount reporter gene staining, embryos were fixed in Flow fix for 1 h at 4°C and processed for X-Gal staining. Images were captured using a Leica DFC 500 digital acquisition system.

Immunohistology. Cryosections (10 μm) were fixed in methanol or Flow fix for 15 min at 4°C, blocked and processed for immunostaining with antibodies for β-galactosidase (Cappel, ICN; no. 55976), sarcomeric myosin (DSHB; no. MF20), α-cardiac sarcomeric actin (Sigma; no. EA-53), or Cx43 (BD Transduction Laboratories; no. 800,000 cells per hindlimb). For direct intracardiac cell injection experiments, dispersed cell preparations from E11.5, E14.5, and E17.5 NCRL donor ventricles were prepared and injected into the left ventricular wall of C57BL6J male mice (30 μl of 300,000 cells in 30 μl of PBS/recipient) as described previously (11, 19, 25).

Approximately 15% of dispersed cells derived from E9.5 embryo stained positive for β-galactosidase (β-gal) activity. [3H]thymidine labeling was performed as described earlier. In some experiments, E11.5 ventricular cells were transfected with 2.5 μg of a lineage tracking triple fluorescent NEMEAD construct (17) using Lipofectamine 2000 (Invitrogen) for 6 h, fed with fresh medium and maintained for 48 h. Identification of CPCs and imaging of Ca2+ transients with fluo 3-AM were performed as described in our previous work (17).

Transmission electron microscopy (TEM). Embryonic ventricles were fixed in 2.5% glutaraldehyde (Canemco-Marivac, Quebec, Canada) in 0.1 M sodium cacodylate (pH 7.2) at 4°C for 2 h, postfixed in 1% osmium tetroxide (Canemco-Marivac) for 2 h, and subsequently processed for routine TEM analysis as reported previously (39). Samples were examined using a JEOL JEM 1230 transmission electron microscope at 80 kV, and images were captured using a Hamamatsu camera.

Cell transplantation and graft analysis methods. For hindlimb cell transplantation model, C57BL6J mice were anesthetized using 2.5% isoflurane. NCRL ventricular cells were injected into the quadriceps (800,000 cells per hindlimb). For direct intracardiac cell injection experiments, dispersed cell preparations from E11.5, E14.5, and E17.5 NCRL donor ventricles were prepared and injected into the left ventricular wall of C57BL6J male mice (30 μl of 300,000 cells in 30 μl of PBS/recipient) as described previously (11, 19, 25).

At the end of 3 or 7 days post cell injections, recipient quadriceps or hearts were collected and fixed in Flow fix. Subsequently, vibratome sections (30 μm) or cryosections (10 μm) were generated and processed for X-Gal, hematoxylin and eosin, or immunohistochemistry as described previously (23). Graft sizes were calculated as the area (mm2) occupied by the X-Gal staining in thin sections using a previously described image analysis method (10).

Analysis of surface electrocardiograms (ECG). Mice were anesthetized using 2.5% isoflurane, and the body temperature was controlled with a rectal probe coupled with homeothermic controller and heating plate (ADInstruments). ECGs were recorded using Bio AMP and Power Lab 8/30 hardware (ADInstruments) at a sampling rate of 1 kHz for a minimum of 10 min. Random frames were used to determine the heart rate, RR, PR, QRS, and QTc durations using Lab Chart 7Pro software (v.7.3.7, ADInstruments).

Statistical analysis. Data are expressed as means ± SE and were considered statistically significant when the difference in mean values between groups had a P value ≤ 0.05. All data comparisons were completed using an unpaired two-tailed t-test or one-way ANOVA. Significance obtained by ANOVA was further subjected to a Tukey’s test for post hoc analysis. All statistical analyses were performed using Graphpad Prism (Graphpad Software, San Diego).

RESULTS

Lineage tracking of Nkx2.5+ myocardial cells during cardiac development. To identify cells derived from primary and secondary heart fields, a Cre-dependent β-galactosidase (lacZ) reporter mouse strain (ROSA26; designated RL) was bred with the Nkx2.5-Cre knockin mouse line [designated NC; (34); Fig. 1A]. The Cre-mediated reporter gene expression was confirmed by lacZ staining in double transgenic offspring (NCRL) throughout development in cardiac crescent, heart tube, OFT, and subsequently in four-chambered hearts at E11.5 stage (Fig. 1, A–E). We further determined the percentage of Nkx2.5+ progenitors in whole embryo (E7.5) or ventricular (E9.5 to neonatal day 1) dispersed cell cultures after X-Gal staining (Fig. 2, A and B). Less than 5% of cells derived from E7.5 embryo stained positive for β-galactosidase (β-gal) activity. Approximately 15% of dispersed cells derived from E9.5 ventricles were positive for reporter gene expression. In con-
trast, the percentage of Nkx2.5<sup>+</sup> cells significantly increased to 60% for E11.5 stage and close to 70–85% for subsequent stages of heart development (Fig. 2B).

Cell cycle dynamics of Nkx2.5<sup>+</sup> cells during cardiac ontogeny. We monitored the G1/S phase transit in dispersed NCRL ventricular cells which were processed for X-Gal staining and in situ [<sup>3</sup>H]thymidine autoradiography. The labeling index (LI) was assessed as the proportion of the total number of X-Gal–positive cells that displayed nuclear [<sup>3</sup>H]thymidine silver grains (Fig. 2A). In case of X-Gal–negative cells (Nkx2.5<sup>−</sup> cells), the LI was monitored using Hoechst 33342 nuclear staining and presence of silver grains. The LI of Nkx2.5<sup>+</sup> cells stayed close to 40–50% between E7.5 and E11.5 stages and thereafter significantly decreased by two- to fourfold in cells derived from subsequent stages of ventricular development (Fig. 2C). In contrast, the LI of Nkx2.5 negative cells (Nkx2.5<sup>−</sup>) stayed close to 20–30% with no significant difference between groups throughout different stages of embryonic heart formation (Fig. 2D). These results indicate that while the percentage of Nkx2.5<sup>+</sup> cell lineage increases, the proliferative capacity of Nkx2.5<sup>+</sup> cell lineage gradually decreases during ventricular development (Fig. 2E).

Residual cardiac progenitor cells in midgestation ventricles exhibit higher cell cycle activity compared with differentiated cardiomyocytes. Dispersed cells from E11.5 and E14.5 NCRL ventricles were used to distinguish CPCs and cardiomyocytes (CMs) by immunostaining with β-galactosidase and MF20 antibodies. In this assay, Nkx2.5<sup>+</sup> cell lineage–derived CPCs and CMs were identified as β-gal<sup>−</sup>MF20<sup>−</sup> or β-gal<sup>−</sup>MF20<sup>+</sup> cell populations, respectively (Fig. 3, A–C). Subsequently, using a combination of β-gal, MF20, and thymidine labeling (Fig. 3, A–D), we found that CPCs exhibit significantly higher cell cycle activity (2- to 4-fold) compared with differentiated myocardial cells from both E11.5 and E14.5 ventricles (Fig. 3E).

Notably, cell cycle activity of both CPCs and CMs from E14.5 ventricles significantly decreased compared with similar cell fractions obtained from E11.5 stage. We next examined for the presence of mitotic figures in cross-sections of E11.5 ventricles using routine TEM. Using the TEM technique, embryonic CPCs can be readily distinguished from the CMs by the absence of myofilaments and by their larger nuclear-to-cell area ratios (Fig. 4A) as described in our earlier reports (17, 39). Nonmyocytes such as endothelial cells and interstitial cells can be distinguished by their smaller size and unique ultrastructural features (Fig. 4B). Furthermore, immunogold labeling analysis of ultrathin sections revealed that undifferentiated CPCs with large nuclear-to-cytoplasmic ratio were positive for Nkx2.5 staining as described in our earlier studies (17, 39). Consistent with the high levels of thymidine-labeling index in CPCs (Fig. 3E), mitotic figures were readily identified in these cells in ultrathin sections at E11.5 stage (Fig. 4, C–F). These data provide evidence that CPCs found in the midgestation hearts retain higher proliferation rates compared with CMs.

Residual cardiac progenitor cells differentiate into functional cardiomyocytes. Using the NCRL lineage tracking in combination with sarcomeric myosin staining method, the percentage of CPCs (β-gal<sup>−</sup>MF20<sup>−</sup>) was found to be twofold higher in E11.5 ventricular cells compared with those of E14.5 stage cultured for a short period (~6 h; Fig. 5, A and B). Further, culturing of E11.5 cells for a 48-h period led to a 2.4-fold reduction in CPC number with a concomitant increase in differentiated myocytes (Fig. 5C). In a second series of experiments, cardiomyogenic differentiation potential of E11.5 CPCs was determined using a previously described real-time live cell imaging method (17). E11.5 ventricular cells were transfected with a triple fluorescent lineage-restricted reporter and Nkx2.5<sup>+</sup> CPCs identified using this method did not exhibit any intracellular Ca<sup>2+</sup> transients (Fig. 5D). However, when the same CPCs were imaged after 48 h in culture, ~50% CPCs differentiated into cells expressing myosin light chain (MLC2v; data not shown) and exhibited synchronized intracellular Ca<sup>2+</sup> transients (Fig. 5D). Collectively, these results suggest that E11.5 CPCs retain cardiomyogenic potential even after chamber specification.

Midgestation ventricular cells can form larger grafts in a hindlimb cell transplantation model. E11.5 or E14.5 ventricular cells from NCRL embryos were injected into the hindlimbs of syngeneic mice and allowed to develop stable grafts over a 3- or 7-day period (Fig. 6A). Positive cell engraftment areas were readily identified in quadriceps after X-Gal staining (Fig. 6B). Cryosections obtained from the engrafted areas revealed several X-Gal–positive cells in spaces between skeletal muscle fibers of recipient legs (Fig. 6C). Examination of hematoxylin and eosin-stained sections under higher magnification did not reveal any evidence of cell fusion between transplanted cells and the skeletal myocytes (Fig. 6D). Graft sizes of E11.5 cells were significantly larger at both time points compared with those obtained with E14.5 cells at 7-day time point (~3.5–5-fold; Fig. 6E).

E11.5 ventricular cells with residual CPCs form larger grafts after direct intracardiac cell transplantation. NCRL ventricular cells from E11.5 and E14.5 stages were directly injected into the ventricular myocardium of nontransgenic recipient mice and allowed to develop stable grafts over a...
7-day period. Subsequently, histological sections obtained from recipient hearts were processed for X-Gal staining (Fig. 7, A–C). Quantification of graft areas suggested that E11.5 ventricular cells can form significantly larger grafts compared with E14.5 ventricular cells (Fig. 7D). All transplanted cells expressed sarcomeric actinin and revealed clear sarcomeric structures that are typically found in differentiated myocytes (Fig. 7, E–H). However, none of the transplanted cells stained positive for a conduction system marker acetylcholinesterase (not shown). In addition, transplanted embryonic ventricular cells adjoining the host myocardium also expressed gap junction protein Cx43 (Fig. 7, I and J). No overt ECG abnormalities were observed in recipient mice after intracardiac transplantation of embryonic ventricular cells (Fig. 7K).

**DISCUSSION**

Spatiotemporal studies on cell cycle dynamics in the developing heart revealed higher proliferation rates in regions corresponding to the ballooning of chamber myocardium compared with low-proliferation zones in the OFT, interventricular septum, and atrioventricular canal (4, 6, 28). However, no studies were designed to monitor cell cycle changes in primary or secondary heart field descendants. Moreover, previous studies did not monitor whether there are any differences in cell cycle kinetics between undifferentiated CPCs and myocytes during embryonic heart development. Here, we used multiple marker analysis on dispersed cells preparations and demonstrated that cardiomyogenic CPCs present in the ventricular myocardium of midgestation embryonic stages still retain a higher proliferative capacity compared with differentiated myocytes. Using two different cell transplantation models, we further demonstrated that E11.5 ventricular cells containing highly proliferative CPCs and differentiated cells can form larger grafts compared with less proliferative E14.5 ventricular myocytes.

A low percentage of Nkx2.5 cells found at early developmental stages is consistent with the fact that neural crest cells and cell types other than heart field progenitors also participate in heart development (35). While higher proliferation rates can certainly account for the gradual increases seen with the Nkx2.5 lineage, contribution of progenitors from other sources to Nkx2.5 cell number also needs further investigation. Although Nkx2.5 promoter activity was shown to be subjected to spatiotemporal regulation in the developing ventricles using a lacZ transgenic approach (27), the lower percentage of Nkx2.5 cells observed at the E11.5 stage cannot be attributed to the promoter downregulation as the cell lineage tracking experiments were performed using a Cre-Lox-based approach in this study.

Some studies showed that assessment of lacZ reporter expression using activity-based assays (e.g., X-Gal staining) may be somewhat less efficient compared with immunostaining-based methodologies (5). Based on this observation, we used the immunostaining method as a more sensitive approach for visualization of lacZ (Nkx2.5+) cells in dispersed cell preparations. Since lineage tracing by β-gal staining alone does not
allow inferences to be made regarding the differentiation status, simultaneous labeling of cells with antibodies specific for β-gal and sarcomeric myosin was used to unambiguously identify CPCs (β-gal+/MF20−) and cardiomyocytes (β-gal+/MF20+) in the present study. Further, a reduction in CPC number and appearance of Ca2+ transients in long-term E11.5 ventricular cultures are consistent with the previously demonstrated myocyte-specific gene expression changes in differentiating CPCs (17).

Given the drastic decreases in proliferation rates of both CPCs and myocytes from E11.5 to E14.5 stages, it is likely that both paracrine signaling as well as gene expression changes may play a critical role in this process (20). Recent work from Srivastava and Olson (33) demonstrated that cardiomyocyte proliferation can be regulated by factors secreted from rapidly expanding fibroblasts via β1 integrin signaling in the embryonic heart (12). While this study did not examine changes in the secretion profiles of fibroblasts at different stages of development, it is possible that such changes in paracrine signals between E11.5 and E14.5 stages could account for one of several mechanisms responsible for cell cycle exit in later stages of heart development. This notion is further supported by the observation that adult cardiac fibroblasts with distinct gene expression profile induced hypertrophy rather than proliferation of embryonic cardiomyocytes in a coculture system (12).

Our findings related to ventricular cell transplantation suggest that a preemptive consideration of donor cell developmental stage and proliferative potential prior to cell transplantation may improve the efficiency of myocardial repair in both experimental and clinical studies. While pharmacological and survival factor treatments have been widely considered to improve grafting efficiency in the myocardial cell transplantation field (14), no studies to date have directly correlated the mitotic potential and or developmental stage of donor cells with the efficiency of myocardial repair. Our findings in this regard are in strong agreement with those obtained from embryonic striatal cell transplants in the adult primate and rat brains (9, 30). It was found that striatal primordial cells from younger donor stages (E14 and E16) can produce grafts with a
significantly higher proportion of striatal-like tissue compared with later developmental stages (E17 or E19) (9). Further, our results on midgestation ventricular cell proliferation kinetics are also in agreement with a recent report which documented a remarkable regenerative ability of midgestation embryonic heart using a genetically determined mosaic model for respiratory chain deficiency (7). In their study, Drenckhahn and colleagues (7) demonstrated that hyperproliferation of healthy

![Graphs showing cell distribution](image)

**Fig. 5.** Relative distribution of embryonic CPCs and CMs and cardiomyogenic differentiation potential of E11.5 CPCs. A–C: percent cell distribution of CPC, CM, and nonmyogenic/nonmuscle cells (NMC) in E11.5 or E14.5 ventricular cells cultured for 6 h (A and B) or 48 h (C); *P < 0.005, CPC vs. CM (ANOVA). D: absence of Ca^{2+} transients in a CPC identified by a lineage-restricted triple fluorescent construct at 12-h time point (top panel) and appearance of Ca^{2+} transients in the same cell (bottom panel) after culturing for 48 h.

**Fig. 6.** Comparison of engraftment efficiencies of E11.5 and E14.5 ventricular cells in a hindlimb cell transplantation model. A: schematic showing steps involved in hindlimb cell transplantation procedure. B and C: micrographs showing Nkx2.5 donor cell engrafted areas (X-Gal+ areas) in a piece of quadriceps (B) or 10 µm cryosections. Bars = 1 mm (B) and 50 µm (C). D: hematoxylin and eosin-stained sections show no evidence of cell fusion between grafted ventricular cells with skeletal myocytes (SM). Bar = 50 µm. E: quantification of engraftment or seeding efficiency of E11.5 and E14.5 donor cells. Data are presented as means ± SEM. n = 3 experiments/each group. *P < 0.05 or **P < 0.005, ANOVA. NCRL: Nkx2.5-Cre and ROSA-lacZ double knockin embryos.

![Graphs showing cell engraftment efficiency](image)
heart cells was sufficient to compensate for the loss of at least 50% of diseased tissue and subsequent formation of fully functional heart after birth. Indeed, our findings on divergent cell cycle kinetics as well as transplantation efficiency of E11.5 ventricular cells underscore the importance of CPCs in post-midgestation heart development and also as optimal donor cells in myocardial repair.

Previous in vitro studies suggested that CPCs isolated from pre-midgestation stages appear to be multipotent or bipotential cells based on their developmental stage. For instance, Flk1/CPCs isolated from headfold embryo stage were shown to generate cardiomyocytes, endothelial cells, and smooth muscle cells when cultured using appropriate cytokines (13). Wu and colleagues (37) showed that CPCs generated from E9.5 stage were positive for Nkx2.5, Isl1, and c-Kit expression and these cells were able to form working myocytes and smooth muscle cells. In contrast, midgestational CPCs (Nkx2.5+ but negative for markers such as MLC2v, ANF, c-Kit, and Isl1) were shown to differentiate into cardiomyocytes or putative conduction system cells in vitro (17). In this study, transplantation of E11.5 ventricular cells containing a large number of CPCs mainly generated grafts containing well-differentiated Nkx2.5+ cardiomyocytes but not other cell types. These data suggest that differentiation potential of embryonic CPCs may be mainly governed by the type of cues surrounding the graft in the host myocardium.

In addition, transplanted embryonic ventricular cells maintained Cx43 expression which is a prerequisite for electromechanical coupling of donor cells to the host myocardium. This result is particularly important due to the fact that absence of Cx43 expression in donor cells was shown to increase the incidence of arrhythmia after intracardiac transplantation (24). Further, absence of ECG abnormalities suggests that transplantation of donor cells containing a large number of CPCs may be a safe alternative for myocardial repair. With the advent of induced pluripotent stem (iPS) cell technology, isolation of patient-specific CPCs is feasible in the near future. Nonetheless, it is important to be cautious about the risks that may emanate from the use of iPS-derived CPCs, including the risk of tumor formation in a human heart and uncertainty about the usefulness of these cells for repair when placed into an unfriendly environment such as hearts with myocardial infarction and heart failure. Hence, additional studies on long-term consequences of iPS-derived CPC transplantation are fully warranted before realization of their clinical potential.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: F.Z., T.F., A.H., and K.B.P. conception and design of research; F.Z., T.F., and A.H. performed experiments; F.Z., T.F., A.H., and K.B.P. analyzed data; F.Z., T.F., A.H., and K.B.P. interpreted results of experiments; F.Z., T.F., and K.B.P. prepared figures; F.Z. and K.B.P. drafted manuscript; F.Z., T.F., A.H., and K.B.P. approved final version of manuscript; A.H. and K.B.P. edited and revised manuscript.

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