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Diffusion Tensor Imaging and Histology of Developing Hearts

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Abstract

Diffusion tensor imaging (DTI) has emerged as a promising method for noninvasive quantification of myocardial microstructure. However, the origin and behavior of DTI measurements during myocardial normal development and remodeling remain poorly understood. In this work, conventional and bicompartamental DTI in addition to three-dimensional histological correlation were performed in a sheep model of myocardial development from third trimester to postnatal 5-months of age. Comparing the earliest time points in the third trimester to the postnatal 5-months group, the scalar transverse diffusivities preferentially increased in both left ventricle (LV) and right ventricle (RV): secondary eigenvalues D_2 increased by 54% (LV) and 36% (RV), whereas tertiary eigenvalue D_3 increased by 85% (LV) and 67% (RV). The longitudinal diffusivity D_1 changes were small, which lead to a decrease in fractional anisotropy (FA) by 41% (LV) and 33% (RV) in 5-months vs. fetal hearts. Histological analysis suggested that myocardial development is associated with hyperplasia in the early stages of the third trimester followed by myocyte growth in the later stages up to 5-months of age (increased average myocyte width by 198%, myocyte length by 128%, and decreased nucleus density by 70% between preterm and postnatal 5 months hearts.) In few histological samples (N=6), correlations were observed between DTI longitudinal diffusivity and myocyte length ($r=0.86$, $P<0.05$), and transverse diffusivity vs. myocyte width ($r=0.96$, $P<0.01$). Linear regression analysis showed that transverse diffusivities are more affected by changes in myocyte size and nucleus density changes than longitudinal diffusivity, which is consistent with predictions of classical models of diffusion in porous media. Furthermore, primary and secondary DTI eigenvectors during development changed significantly. Collectively, the findings demonstrate a potential role for DTI to monitor and quantify myocardial development, and potentially cardiac disease.

Graphical abstract

Diffusion tensor imaging (DTI) was performed in a fetal sheep model of heart development. Findings were supported with bicompartamental DTI and histology. Results indicate that diffusivity

increased and anisotropy decreased during development, which correlated with histological measurements of increased myocytes size and decreased nucleus (or cell) density. Acronyms used are: fractional anisotropy (FA), mean diffusivity (MD, 10^{-3} mm²/s), longitudinal diffusivity ($D_{||}$, 10^{-3} mm²/s), transverse diffusivity ($D_{\perp}$, 10^{-3} mm²/s) helix angle (HA, °), secondary eigenvector angle (E2A, °), histology (Hist, scale bar 50 μ m) with myocytes (black), extracellular space (green), and nuclei (blue).

Keywords

DTI; fractional anisotropy; mean diffusivity; principal diffusivities; helix angle; sheet angle; preterm lamb heart

Introduction

Preterm birth complications are the leading cause of death among children under 5 years of age (1). Because the fetus' lungs mature within the final weeks of pregnancy, a preterm infant may develop acute respiratory failure that may evolve into chronic lung disease (bronchopulmonary dysplasia, BPD). BPD is histologically characterized by alveolar simplification, persistent muscularization of resistance pulmonary arterioles, and pulmonary hypertension (2). These structural disruptions of the pulmonary vasculature in BPD lead to development of persistent pulmonary hypertension that is refractory to treatment. One of the long-term consequences of persistent pulmonary hypertension in former preterm children is heart failure. To date, post mortem histology remains the primary method for characterizing myocardial structural development during normal growth or associated with persistent pulmonary hypertension. Therefore, noninvasive imaging methods to characterize myocardial structural information are highly desirable.

MR diffusion tensor imaging (DTI) (3) has been used to noninvasively measure anisotropic diffusion, and thus the magnitude and spatial orientation of microstructural organization of

tissues. DTI has emerged as a preferred technique for noninvasive quantifications of myocardial structures. Although feasibility of *in vivo* cardiac DTI has long been demonstrated (4–6), recent advances in both gradient hardware and imaging technologies have made *in vivo* cardiac DTI more widely used (7–10). The principal direction of diffusion anisotropy (i.e., the DTI first eigenvector) has been directly correlated to myocardial fiber orientation (11), and used to characterize myocardial fiber structure in human (4), small (12), and large animals (13). DTI secondary and tertiary eigenvectors have been suggested to correlate with local myocardial sheets and sheet-normal directions, respectively (14,15). Recently, changes in the relative distributions of the secondary eigenvector from diastole to systole associated with hypertrophic cardiomyopathy have been observed via *in vivo* imaging (16), but the extent to which they capture the underlying myocardial sheet structural changes remains unclear (17,18).

While heterogeneity of microstructure is well-established in cardiac and other tissues, the origin and behavior of observed DTI changes during cardiac development remain poorly understood. Specifically, how changes in DTI parameters correlate with changes in myocardial tissue components, such as increased myocyte size and density that are commonly observed in normal growth and cardiomyopathy, are incompletely understood. Compartmental analysis has been helpful to interpret the MRI signal in tissues especially the brain (19,20), with few studies reporting it in hearts (21,22). In MRI, a compartment is broadly defined as a moiety of spins that share the same attributes, which can be physical location (e.g., intracellular or extracellular), macromolecular interaction (bound or free water), or other means that give rise to their distinct properties. To make analysis of DTI signals tractable, compartmental modeling often examines the models under extremes of exchange such as the slow and fast exchange limits (23). In the mostly-used slow exchange limit, the aggregate MR signal is the sum of signals of the constituents weighted by their compartment sizes. However, limited independent validating methods in cardiac DTI during tissue remodeling complicates interpretation of the observed DTI parameters.

The preterm lamb model (2) offers unique opportunities to study development of the normal heart (e.g., myocyte growth) and in disease (e.g., persistent pulmonary hypertension) (24). Although costly and laborious, the fetal lamb model is unique for investigation of cardiovascular and lung development and mechanisms of disease pathogenesis because it recapitulates human conditions of fetuses of similar premature gestation age (25,26). Growth of the perinatal heart is mainly characterized by myocyte hyperplasia and postnatally by myocyte growth (25,27–29). During the third trimester of gestation, cardiac myocytes enlarge and often contain two or more nuclei per cell (30). Fetal cardiomyocytes have fewer and less organized myofibrils, with poorly developed sarcoplasmic reticulum and transverse tubular system compared to adult cardiomyocytes (31). Therefore, the preterm lamb model offers a unique validating model for DTI analysis. For this study, we aimed to (a) characterize normal myocardial development in fetal lamb hearts of different gestational ages and from term lambs, using DTI, and (b) provide insights on how the observed DTI changes correlate with tissue microstructure, using bicompartamental DTI and 3D confocal microscopy.

Methods

Preterm Lamb Model and Specimen Collection

Fetal lambs were delivered via cesarean section. Lambs were heparinized and then given 3 ml KCl intravenously moments before euthanasia followed by a lethal injection of concentrated pentobarbital solution (Beuthansia solution, Ovation Pharmaceuticals, Inc., Deerfield, IL). The heart was excised and placed in 250-400 mL cold phosphate buffered saline (PBS), with 10 mEq of KCl added. The heart was perfused through the coronary arteries, then placed in 4% buffered formalin and stored at 4°C for at least 24 hours prior to imaging.

Five groups were studied and separated based on their gestational ages: (i) D120 (N=6) delivered at 115 ± 9 d (average $\pm$ SD), (ii) D130 (N=8) delivered at 132 ± 3 d, (iii) D140 (N=6) delivered at 142 ± 2 d, (iv) D150 term-born (N=6) delivered at 150 ± 0 d, and (v) 5m (N=8) term-born but sacrificed at 5 months of age (equivalent to a 6-year old human).

MRI Acquisition and Analysis

MRI experiments were performed on a Bruker 7.0 T horizontal bore MRI scanner (Bruker Biospin Inc., Ettlingen, Germany) interfaced with a 12.0 cm-diameter actively shielded gradient insert capable of producing magnetic field gradients of up to 600 mT/m. Each heart specimen was placed in a sealed container filled with susceptibility matching fluid (Fomblin, Solvay Solexis, NJ, USA). Depending on the sample size, quadrature volume coils with different inner diameters (50, 72, or 85 mm) were used for signal transmission and reception such that the signal-to-noise ratio (SNR) of the non-diffusion weighted reference image (I_0) for different heart sizes was above 80 for the same scan time.

Conventional DTI acquisition was performed using a standard multi-slice diffusion-weighted spin echo sequence with the following imaging parameters: 1500/30 ms TR/TE, 64×64 matrix size with 0.7-1.5 mm in-plane resolution, 5 short-axis slices, with 2.0 mm thickness. Diffusion was encoded along a set of 12 optimized gradient directions (32), using a pair of trapezoidal gradient pulses of 0.25 ms rise time, 5.00 ms duration (δ), and 15.00 ms separation (Δ), equivalent to a nominal b-value of 1500 s/mm^2 . The total scan time for each specimen was approximately 30 min.

Post-processing computation was performed using custom-written scripts in MATLAB (Version R2015a, Mathworks, MA) as described previously (33). Briefly, diffusion tensors were estimated on a voxel-by-voxel basis from the diffusion weighted images and diagonalized to obtain the 3 eigenvalues (D_1 , D_2 and D_3 , in sorted descending order and commonly referred to as primary, secondary, and tertiary diffusivities, respectively) and their associated eigenvectors. The primary eigenvalue at each voxel reflects the diffusion coefficient longitudinal to the fibers, whereas the secondary and tertiary eigenvalues represent the diffusion coefficient transverse to fibers in the direction of the sheet and sheet-normal, respectively. The diffusion tensor eigenvalues were then used to obtain the mean diffusivity (MD) and fractional anisotropy (FA), which represent the magnitude of diffusion and degree of anisotropy. Heart images were manually segmented using Amira (Visualization Science Group, Bordeaux, France) to obtain binary masks of the right (RV)

and left ventricles (LV). The scalar DTI quantities (D_1 , D_2 , D_3 , and FA) were averaged from regions-of-interest (ROIs) covering the whole segmented tissue area in each ventricle.

DTI orientation maps were obtained from the primary ($\hat{E}_1$) and secondary ($\hat{E}_2$) eigenvectors. A local coordinate system at each voxel was defined and constructed using a distribution of potentials, described by the Laplace Dirichlet method for structural definition (34), and calculated using FEBio software suite (35). At each imaging voxel, the radial ($\hat{a}$) (endocardium towards epicardium), longitudinal ($\hat{l}$) (apex to base), and circumferential ($\hat{c} = \hat{a} \times \hat{l}$) directions were obtained. The helix angle (HA) is defined as the angle between the projection of $\hat{E}_1$ onto the tangent plane to the epicardium wall ($\hat{l} - \hat{c}$) and the direction of $\hat{c}$, ranging from -90° (pointing toward apex) to $+90^\circ$ (pointing toward base). The angle between the projection of $\hat{E}_2$ onto the radial plane ($\hat{l} - \hat{a}$) and the local $\hat{a}$ direction was termed the secondary eigenvector angle (E2A), which is assumed to follow the local sheet direction (14,36,37). Positive E2A angles were inclined toward the base and negative toward the apex.

Voxel-wise ventricular averages of HA measurements were obtained from manually segmented LV and RV masks. Transmural rotation of HA for the whole ventricle was obtained by fitting a linear function to the normalized transmural scatter in each slice, and the slopes from 5 slices were averaged for each heart. Further, the HA zones were stratified into the left handed fibers (LHF) ($-90^\circ < HA < -30^\circ$), circumferential fibers (CF) ($-30^\circ < HA < +30^\circ$), and right handed fibers (RHF) ($+30^\circ < HA < +90^\circ$) as this stratification was previously shown to characterize cardiac disease (38–40). Voxel-wise E2A values were averaged over the whole ventricles and stratified to positive, negative, and absolute measurements.

Bi-Compartmental DTI Analysis

To elucidate the source of the observed DTI measurements, bicompartamental DTI experiments (22), consisting of 48 gradient directions and 12 b-values ranging from 100-5000 s/mm² were conducted in 5 hearts randomly selected from each D130 and 5m groups, respectively. To distinguish the hearts used for bicompartamental analysis, we will term the subgroups as D130c and 5mc where “c” refers to compartmental analysis. Other imaging parameters were similar to conventional DTI acquisitions but scan time was approximately 9 hours per heart.

The signal intensity I_j in an encoding direction j was normalized to I_0 and fitted to a slow-exchange bi-exponential model consisting of 2 diffusion tensors, arising from fast (D_f) and slow diffusion (D_s) behaviors (22):

$$I_j/I_0 = (1 - f) \exp(-b \mathbf{g}_j^T \cdot \mathbf{D}_f \cdot \mathbf{g}_j) + f \exp(-b \mathbf{g}_j^T \cdot \mathbf{D}_s \cdot \mathbf{g}_j) \quad [1]$$

where f is the volume fraction of the slow compartment, and $\mathbf{g}_j$ is the diffusion encoding unit vector. Nonlinear least square fitting routine of Eq. [1] containing 13 unknowns (6 unique tensor elements for each component plus the fraction f) was implemented in MATLAB. Cholesky parameterization of the diffusion tensor (41) was used to ensure positive-definite

tensor elements. Similar procedure to conventional DTI analysis was performed to obtain the scalar principal diffusivities and FA in the LV for each fast and slow compartment. Median filtering (width of 3 pixels) was applied to the parametric maps to remove isolated noisy voxels.

Confocal Microscopy

Transmural biopsies with a diameter of 5 mm were taken from the LV free wall in six randomly selected hearts spanning the third trimester: D100 (two hearts), one heart of each D130, D140, D150, and term 5m. Biopsies were then put into a 30% sucrose solution for >4 hours to avoid tissue damage during the subsequent freezing process. Before cryosectioning, biopsies were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, Netherlands). Sections parallel to the epicardial surface of 80 μm thickness were cut at -24°C using a cryotome (CM 1950, Leica AG, Wetzlar, Germany). Slices from the subepicardium, mid-wall, and subendocardium were obtained from each biopsy. After cryosectioning, OCT remnants were removed by rinsing the tissue slices three times with PBS, containing in mM: 137 NaCl, 2.7 KCl, 10 Na₂HPO₄, and 1.8 KH₂PO₄. Extracellular space constituents (including myocyte borders) were labeled using CF488A-conjugated wheat germ agglutinin (WGA, 29022, Biotium, Hayward, CA, USA) applied at a concentration of 40 $\mu\text{g/mL}$ in PBS for at least 6 hours. WGA is an established marker for glycoconjugates in the extracellular matrix that binds to cell membranes, collagen strands, and vessel walls (42–44). Additionally, nuclei (myocytes, endothelial cells, fibroblasts, etc) were stained with 1 μM 6-diamidino-2-phenylindole (DAPI, D3571, Life Technologies). All sections were mounted in Fluoromount-G (#17984-25, Electron Microscopy Science, Hatfield, PA, USA) on a glass slide and scanned using a Leica SP8 confocal microscope with a 40x oil immersion lens (numerical aperture 1.3). Two types of scans were performed in each slide, including (i) 2D tile scans with a pixel size of $0.2\ \mu\text{m} \times 0.2\ \mu\text{m}$ spanning $1000\ \mu\text{m} \times 1000\ \mu\text{m}$ FOV, and (ii) 3D scans with a voxel size of $0.2\ \mu\text{m} \times 0.2\ \mu\text{m} \times 0.2\ \mu\text{m}$ covering a volume of approximately $205\ \mu\text{m} \times 205\ \mu\text{m} \times 50\ \mu\text{m}$. DAPI was excited with a 405nm laser, and WGA-CF488 with a 488nm laser. To minimize depth-dependent intensity attenuation, linearly increasing laser powers were used as described recently (45).

Quantitative analysis of cell size and nucleus density from DAPI and WGA volumes were performed in MATLAB. Nucleus density was calculated by a series of morphological analysis including applying (i) intensity-based threshold (mode + 2 SD) to segment the DAPI signal, (ii) median filtering with a circular kernel of 3 pixels, (iii) erosion then dilation operations to remove small pixels while maintaining the size of nuclei, and (iv) connected-component analysis to detect individual nuclei. Nucleus density (ND, units /mm²) was obtained by dividing the number of detected nuclei by the overall tissue area.

To characterize myocyte hypertrophy, estimates of myocyte size (length and width) was obtained from the 3D confocal images (fused WGA and DAPI) by manually measuring the average length and width of 10 randomly selected myocytes (each myocyte was measured in 3-5 different locations then averaged). Only myocytes with clear and defined borders in the 3D stacks were evaluated. To characterize hyperplasia, the LV free wall transmural width

was measured from MR images and divided by the average myocyte width to estimate approximate myocyte count in the transmural wall.

Correlation between Confocal Microscopy and Conventional DTI

To quantify the utility of DTI's scalar parameters to detect myocardial microstructural changes, the same 6 heart specimens that underwent confocal microscopy were analyzed by manually identifying 3 transmural regions in the LV free wall close to the biopsy locations and then averaged. Scatter plots of DTI's D_1 vs. myocyte length and transverse diffusivity ($D_{\perp}$, average of D_2 and D_3) vs. myocyte width were obtained. To characterize the effects of changing nucleus density (ND) on the DTI parameters, scatter plots were also obtained for DTI's scalar parameters vs. ND.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (Graph-Pad Prism 5.0a, GraphPad Software Inc., La Jolla, CA). Variables were reported as mean and standard error of the mean (SEM). The DTI parameters measured in the current study, including the scalar and vectorial information, were compared as a function of gestation timeframe via analysis of variance. In the Bonferroni-corrected post hoc tests, corrected $p < 0.05$ was considered statistically significant. For bicompartiment DTI analysis, unpaired t-test was used to compare the D130c to 5mc groups with $p < 0.05$ considered statistically significant. Linear regression analyses were used to quantify the association between DTI scalar parameters (averaged from three transmural locations in each heart) and the corresponding myocardial microstructural information from confocal microscopy (averaged from the three transmural sections in each heart).

Results

Conventional DTI Analysis

Figure 1 shows representative gross pictures of maturing lamb hearts obtained using identical fixation procedure. DTI parametric FA and MD maps show that developing hearts become less anisotropic and less restrictive to water diffusion. The appearance of HA and E2A maps is consistent with previous DTI studies (16,46,47). HA maps appear qualitatively similar among groups, while E2A showed qualitative differences during development but not with consistent patterns.

Figure 2 and Tables 1 and 2 summarize the group-averaged changes of the DTI scalar in the left and right ventricles. Qualitatively, the transverse diffusivities changed gradually in the earlier points in the third trimester, which then became more pronounced at 5m of age. Specifically, comparing the youngest preterm group D120 to the term 5m group, the scalar transverse diffusivities preferentially increased in both LV and RV: D_2 increased by 54% (LV) and 36% (RV), whereas D_3 increased by 85% (LV) and 67% (RV). The increased longitudinal diffusivity D_1 was not statistically significant for most cases, except for D140 vs. 5m in LV, D120 vs. 5m and D140 vs. 5m in RV, where the average increase was 15%. Collectively, the changes in transverse diffusivities resulted in decreased FA by 41% (LV) and 33% (RV) from D120 to 5m.

The directional DTI parameters shown in Fig. 1 and 3 are consistent with previous cardiac DTI studies (12,16,38,47,48). Compared to D120, HA distribution in LV of 5m significantly shifted toward circumferential orientation (17% increase) at the expense of significant reduction of LHF (by 44%) (Table 1), while the HA slope decreased by 28% only in the RV (Table 2). No significant changes in the absolute HA in both ventricles were found, although there was a trend of increasing mean HA in the RV at 5m (Fig. 3b and Table 2). The E2A histograms (Fig. 3c and d) symmetrically rearranged to a relatively more vertical alignment in 5m compared to D120. The absolute E2A increased by 50% and 92% during development in both the LV and RV, respectively, from D120 to 5m groups, with the changes equally driven by the negative and positive E2A components in both ventricles (Tables 1 and 2).

Compartmental DTI Analysis

To investigate the origin of the scalar DTI parameter changes during development, we applied compartmental DTI analysis to a preterm D130c and term 5mc hearts. Figure 4 shows signal decays longitudinal and transverse to myocytes (in the circumferential fibers region) encoded up to a b-value of 5000 s/mm² for preterm D130c and term 5mc hearts. Clearly, the signal decay from preterm and term hearts are more separated when diffusion encoding was applied transverse to myocytes (Fig. 4b) compared to the longitudinal (Fig. 4a) direction. Figure 5 shows representative bicompartiment DTI scalar and orientation maps for both fast and slow compartments. Consistent with a previous bicompartiment cardiac DTI study (22), the slow FA map is globally higher than the fast FA, and both fast and slow FA decreased during development, which is also consistent with the conventional DTI findings (Fig. 1). The mean diffusivity maps also show slight increase at the 5mc compared to the preterm heart. The helix angle maps were remarkably similar between fast and slow compartments, and show little difference between the different time points. Table 3 summarizes the group-averaged bicompartimental DTI findings. As in classical DTI, there was a preferential increase in both fast and slow transverse diffusivities (D_2 and D_3), and insignificant change in the D_1 components, which resulted in decreased FA from D130c to 5mc. The volume fraction of the slow component decreased from 37% at D130c to 21% at 5mc.

Confocal Microscopy

Confocal microscopy images for representative heart specimens are shown in Fig. 6. DAPI images revealed smaller nuclei and higher density in the fetal hearts (D100 and D130) compared to term hearts (D150 and 5m). Zoomed 3D confocal images (overlay of DAPI +WGA) in Fig. 6 clearly display increased myocyte size (black spaces) and reduced nucleus density (less blue stain) in term (D150 and 5m) compared to fetal hearts (D100 and D130). Table 4 summarizes the quantitative analysis from the 3 sections of each heart. For example, compared to the D130 heart, tissue from term 5m hearts had decreased nucleus density (70%), whereas myocyte length and width increased by 119%, and 200%, respectively. Also shown in Table 4 the ratio of free-wall thickness (from MRI) to average myocyte width (from confocal microscopy) for selected fetal and term hearts. Relatively more myocytes fill the transmural wall at earlier time points in fetal hearts during the third trimester (D100-D140) compared to term hearts (D150 and 5m).

Correlation between Conventional DTI and Confocal Microscopy

Scatter plots between conventional DTI analysis and confocal-based microstructural parameters, together with fitted linear models, are shown in Fig. 7. Despite the small sample size (N=6 hearts), strong correlations were observed between DTI D_1 vs. myocyte length (Pearson $r=0.86$, $P<0.05$), $D_{\perp}$ vs. myocyte width ($r=0.96$, $P<0.01$), $D_{\perp}$ vs. ND ($r=-0.99$, $P<0.001$), but insignificant correlation between D_1 vs. ND ($r=-0.80$, $P=0.06$). Comparing the slopes of Fig. 7a to 7c and 7b to 7d, myocyte size and nucleus density changes are more reflected in $D_{\perp}$ compared to D_1 .

Discussion

The DTI scalar parameters reveal that fetal hearts during the third trimester of gestation and beyond become less anisotropic and less restrictive to water diffusion. DTI fiber orientation changes were detected in the left-handed and circumferential fibers fractions and helix angle slopes in the LV and RV, respectively. The secondary eigenvector-based E2A measurement was also sensitive to myocardial development in both ventricles. The scalar DTI changes are corroborated by analyses of confocal microscopy images (Table 4), which suggest that heart growth in the earlier points of third trimester is characterized by hyperplasia (higher nucleus density per unit area) and later by hypertrophy (anisotropic myocytes growth), consistent with previous reports in developing lamb hearts (25,29). Despite the limited number of histological measurements, the observed correlations between DTI scalar diffusivities and microstructural parameters from confocal microscopy (Fig. 7) suggest that $D_{\perp}$ is more sensitive to changes in myocyte size and nucleus density than D_1 .

Myocyte growth is likely to be the cause of the observed increases in D_2/D_3 (and reduction in FA). For example, the effective transverse diffusion coefficient ($D_{\perp}$) in parallel cylindrical cells increases with decreased surface-to-volume ratio ($\frac{S}{V}$) and decreased tortuosity in the short and long diffusion-time limits, respectively (49–51). For myocyte diameters ranging from 6 to 18 μm , and with the fixed diffusion time used in the current study $\approx 13 \text{ ms}$, our DTI experiments are in the mid-range of the 2 limits. Nonetheless, a voxel containing a higher density of smaller cells as seen in the fetal heart will have higher surface-to-volume ratio and tortuosity, which in both limits will result in reduced apparent diffusion coefficient compared to a voxel with a smaller number but larger cells. This model also provides a plausible explanation why D_2/D_3 is larger in the RV compared to the LV in the prenatal ages, which is likely because myocytes in the fetal RV are larger than myocytes in the LV (25,29).

For the sake of illustration, suppose that in the short time limit, a voxel containing packed (infinitely long) cylinders of equal radius (r) occupying a volume fraction (ϕ) and ignoring boundaries relaxation, $D_{\perp} \propto D_0 \left[1 - c \sqrt{D_0 t \frac{S}{V}} \right]$, where D_0 is the intrinsic free diffusivity of the medium without restriction effects, $\frac{S}{V} = \frac{2}{r}$ for spins inside packed cylinders (or $\frac{2}{r} \frac{\phi}{1-\phi}$ for spins outside the cylinders), and c is a constant (49–51). The model predicts that $D_{\perp}$ inside packed cylinders will increase by 55% when r increases from 6 to 18 μm (assuming longitudinal diffusivity $D_1 = D_0 = 1 \times 10^{-3} \text{ mm}^2/\text{s}$), which is a good first-order approximation to the observed ~30-80% increase in the experimental D_2 and D_3 for both LV and RV

(Tables 1 and 2). However, the packing model predicts that longitudinal diffusion parallel to the cylinder's main axis is independent of the diameters or packing density changes, which is not entirely true in this study. The small (~15%) changes in D_1 , yet significant in few cases, between fetal and term hearts suggests that other mechanisms may play a role in the observed D_1 changes such as imperfectly parallel myocytes, finite myocytes length, or reduced nucleus density.

Bicompartmental exchange models are often used as a tool to elucidate the observed changes in scalar DTI parameters (52). Qualitatively, the diffusion-induced signal attenuation curve (Fig. 4) is clearly non-monoexponential, especially in the transverse direction to myocytes in fetal hearts. Quantitative analysis revealed that both slow and fast diffusivities increased in similar proportions during heart growth (Table 3). Since the packing model described above can be extended to multiple compartments (50), increased cell packing will result in reduced diffusivities in both compartments. Due to limited histological samples and relative noisiness of the volume fraction estimates from the biexponential fits, direct correlation between volume fractions obtained from confocal microscopy (e.g., WGA stain) and biexponential DTI did not reveal discernable trends (data not shown). Since typical *in vivo* cardiac DTI acquisitions use relatively small b-values (~400 s/mm²), we used retrospective single-exponential DTI fitting to compare scalar DTI parameters at low and high b-values (400 s/mm² vs. 2000 s/mm²). Supplementary Fig. S1 presents that for the same region of interest taken in the LV, all scalar diffusivities and FA trended in similar fashions between the two b-values, which suggest that cardiac growth effects are robust to the choice of the nominal b. However, direct comparison between scalar DTI parameters on fixed specimens and the beating heart is not straightforward. In this study fixed specimens were imaged using spin echo sequence with monopolar diffusion encoding gradients, whereas *in vivo* cardiac DTI is usually achieved using either stimulated echo mode (mostly with monopolar gradients and long mixing time) (8,53) or spin echoes with motion compensated gradients (10,54). Nonetheless, *in vivo* cardiac DTI at low b-values can be affected by changes in microcirculation flow speed and fraction (55,56). Since developing hearts are likely to have changing microvascular flow due to evolving ventricular pressure and angiogenesis (57), microcirculation effects should not be ignored in the context of *in vivo* cardiac DTI of developing hearts.

The DTI orientation parameters also show evidence of fiber and E2A (presumably sheet) changes during heart development. Functional implications of such changes are currently unclear, albeit it can be postulated that those differences are related to changes in heart size or pressure between fetal and term groups. For example, the changes of the RV's HA slope could be related to pressure changes between fetal and term circulation, as reported in RV pressure overload study (58). The observed increase in E2A during development (Fig. 3c and d) may be related to ventricular growth, which is consistent with reported trends between the diastolic and systolic states of healthy hearts (16). However, based on the suggestion that sheet structure forms after birth (59), the presence of sheets may not be the only factor affecting the organized appearance of E2A. Future studies will directly correlate the DTI-based E2A measurements with sheet structures visualized via advanced 3D confocal microscopy staining and processing techniques (45,60).

Previous DTI studies of developing prenatal and postnatal hearts that reported different trends (13,61–63). Human fetal studies reported progressively increasing FA and nearly no coherent fiber organization in fetal hearts taken in early points of the second trimester compared to term hearts (61,62). A pig DTI study reported increasing FA in mid-gestation hearts that decreased after birth, with clearly detectable and coherent fiber organization in all fetal and term ages (13). Another DTI study of postnatal myocardial development in rats showed that FA progressively decreased, and diffusivities increased, from postnatal day 2 to 28 (63). In our study, a highly organized myocardial fiber orientation is present as early as 100 days gestation (early third trimester), with progressively decreasing FA and increasing D_2/D_3 from the earliest time points in the third trimester to the term-born and older hearts (Tables 1 and 2, and Fig. 1 and 2). Our results are consistent with the rat DTI study (when comparing D150 to 5m), but in contrast to the human and pig studies. The discrepancies can be attributed to differences in species, gestational time-points, or specimen fixation procedure. Myocytes and supporting structures in the second trimester of gestation in human and pig may be less organized or contain less barriers to water diffusion compared to the tightly-packed myocytes in the third trimester, which could explain the low FA at younger gestational ages. Another possibility for the discrepancy is that DTI scalar and orientation parameters depend on the cardiac state (systole vs. diastole) (36,46,64). A difficult, if not impossible, challenge is to controllably prepare human heart specimens as quickly or consistently as the heart of experimental animals. The fetal pig hearts in the aforementioned study were not perfused with potassium chloride, whereas the postnatal hearts were perfused with potassium chloride. In our study, the cardiac arrested state (diastole; following potassium chloride injection) and the fixation procedure are standardized for all hearts.

In conclusion, our results indicate that heart tissue remodeling during normal development in sheep manifests in progressively increased DTI transverse diffusivities, decreased FA, distinctive fiber changes in the ventricles, and remodeling in the secondary eigenvectors (which is presumably associated with sheets) during the third trimester and beyond. Bicompartamental DTI analysis revealed that the changes are mediated by similar diffusivity changes in the compartments. Additionally, our findings suggest that the behavior of the transverse diffusivities reflect myocyte density and size, as predicted by a theoretical model of diffusion in porous media (49,50). Our results point at a potential role for DTI in detecting myocyte hypertrophy, for example in the heart of former preterm infants who have bronchopulmonary dysplasia and subsequently develop persistent pulmonary hypertension, which is a national pediatric health problem that has costs for the affected individuals, their families, the health care system, and the educational system (24,26,65).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviation

DTI	Diffusion tensor imaging
FA	fractional anisotropy
MD	mean diffusivity
D₁	longitudinal diffusivity
D₂, and D₃	transverse diffusivities
HA	helix angle
E2A	secondary eigenvector sheet angle
WGA	wheat germ agglutinin
DAPI	6-diamidino-2-phenylindole

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Figure 1.

From top to lower row, representative gross pictures and DTI maps of developing lamb hearts, including scalar fractional anisotropy (FA), mean diffusivity (MD), fiber helix angle (HA) and E2A (obtained from the secondary eigenvector). Fetal hearts at 120 (D120), 130 (D130), 140 days (D140) post gestation, and term 150 (D150) and 5-month (5m) old hearts are shown. Units of MD ($\times 10^{-3} \text{ mm}^2/\text{s}$), and HA and E2A (degrees).

Figure 2.

Groups averaged DTI scalar measurements (mean $\pm$ SEM) taken from regions of interest in the left (LV, top row) and right ventricles (RV, bottom row). Asterisks (*) denote Bonferroni corrected $P < 0.05$ compared to 5-months group. Primary (D₁), secondary (D₂), tertiary (D₃), and fractional anisotropy (FA) are shown. Units of diffusivities ($\times 10^{-3}$ mm²/s).

Figure 3. Group-averaged histograms of myocardial HA (a, b) and E2A (c, d) angles measured from 5 slices in each heart for the LV and RV, respectively. Lines and error-bars represent (mean $\pm$ SEM) of the respective bin. For clarity of presentation, only the averaged histograms from 3 groups are shown for D120 (N=6), D150 (N=6), and 5m (N=8).

a

b

Figure 4.

Normalized diffusion-induced signal attenuation in regions of interest taken from the circumferential fiber region of preterm 130-days (D130) and term 5-months (5m) hearts for b-value up to 5,000 s/mm^2 . Bi-exponential fitting (solid line) is shown for 2 encoding directions (a) longitudinal, and (b) transverse to myocytes.

Figure 5.

Representative maps obtained from bicompartiment DTI showing maps of fast (a, b) and slow (c, d) fractional anisotropy, fast (e, f) and slow (g, h) mean diffusivity (MD), and fast (i, j) and slow (k, l) helix angle (HA) maps from 130-days preterm and 5-month old hearts, respectively. Units of MD ($\times 10^{-3} \text{ mm}^2/\text{s}$), and HA (degrees).

Figure 6.

Confocal images from fetal lambs at 100- and 130-days, full term (at 150-days, or D150), and postnatal term 5-months (5m) hearts. Composite tile-scans for DAPI (top row, nuclei stain in blue), WGA second row (mainly extracellular space stains in green) are shown for 1 mm $\times$ 1 mm tissue area, in addition to zoomed longitudinal (third row) and transverse (lower row) views (overlaid DAPI and WGA) taken from 3D acquisitions with an image resolution of 0.2 $\mu\text{m} \times 0.2 \mu\text{m} \times 0.2 \mu\text{m}$. Scale bar is 50 μm .

Figure 7.

Scatter plots of DTI scalar longitudinal (D_1) vs. myocyte length (a) and nucleus density (b), transverse diffusivities (average D_2 and D_3) vs. microstructural myocyte width (c) and nucleus density (d). Data points represent the mean and SEM of 3 measurements from each heart that underwent both DTI and confocal microscopy. Linear regression (solid lines) and the 95% confidence interval (dashed lines) are shown in addition to the coefficient of determination (R^2) and its associated P value.

Table 1

Left ventricular group-averaged (mean±SEM) DTI principal diffusivities (in ranked order), slope of helix angle (HA) from linear regression, mean HA, and mean E2A (absolute, positive, and negative) obtained from developing hearts at preterm ages of 120 (D120), 130 (D130), 140 (D140), full term (D150), and term at 5-months of age. Units for diffusivities ($\times 10^{-3}$ mm²/s), whereas HA and E2A (°). Asterisks (*) denote Bonferroni corrected $P < 0.05$ compared to 5-months group.

Parameter	D120 (N=6)	D130 (N=8)	D140 (N=7)	D150 (N=6)	5m (N=8)
D ₁	0.86±0.02	0.87±0.01	0.80±0.04*	0.96±0.01	0.92±0.05
D ₂	0.37±0.02*	0.43±0.02*	0.40±0.01*	0.48±0.01*	0.57±0.02
D ₃	0.26±0.01*	0.32±0.02*	0.28±0.04*	0.38±0.01*	0.48±0.02
FA	0.58±0.02*	0.52±0.02*	0.49±0.01*	0.48±0.01*	0.34±0.01
Slope HA	104±18	119±8	117±9	119±4	113±4
Mean HA	0.4±4.9	-8.4±3.3	-3.9±1.8	-8.1±2.3	-6.7±1.2
RHF%	30±1	29±2	28±1	28±2	28±1
LHF%	18±1*	18±1*	19±1*	12±1	10±1
CF%	53±2*	53±1*	53±1*	59±2	62±1
Abs E2A	21±1*	23±2*	27±2	21±2*	30±3
Pos E2A	21±1	21±2*	26±2	21±2*	29±3
Neg E2A	-20±2*	-22±2	-27±2	-21±2*	-30±3

Table 2

Right ventricular group-averaged (mean±SEM) DTI principal diffusivities (in ranked order), slope of helix angle (HA) from linear regression, mean HA, and mean E2A (absolute, positive, and negative) obtained from developing hearts at preterm ages of 120 (D120), 130 (D130), 140-days gestation (D140), full term (D150), and term at 5-months of age. Units for diffusivities ($\times 10^{-3}$ mm²/s), whereas HA and E2A (°). Asterisks (*) denote Bonferroni corrected $P < 0.05$ compared to 5-months group.

Parameter	D120 (N=6)	D130 (N=8)	D140 (N=7)	D150 (N=6)	5m (N=8)
D ₁	0.84±0.02*	0.89±0.01	0.82±0.02*	0.94±0.01	0.96±0.03
D ₂	0.45±0.01*	0.52±0.02*	0.48±0.02*	0.53±0.01*	0.61±0.02
D ₃	0.30±0.01*	0.37±0.01*	0.39±0.01*	0.39±0.02*	0.50±0.02
FA	0.49±0.00*	0.44±0.01*	0.40±0.01*	0.43±0.02*	0.33±0.01
Slope HA	82±6*	75±7*	76±3*	75±5*	59±9
Mean HA	3.9±2.0	6.1±2.1	7.5±3.0	3.8±2.2	11.4±2.5
RHF%	18±1	22±1	21±2	16±1	20±2
LHF%	12±3	8±1	10±1	8±2	8±2
CF%	70±3	70±1	70±2	76±2	72±4
Abs E2A	25±3*	33±2*	34±1*	30±2*	48±3
Pos E2A	25±3*	32±2*	33±2*	28±3*	46±4
Neg E2A	-21±3*	-28±3*	-35±1*	-28±3*	-49±3

Table 3

Group-averaged (mean $\pm$ SEM) bi-compartment scalar DTI parameters from the left ventricles of preterm 130-days gestation (D130c) and from full term hearts at 5-months of age (5mc). The principal diffusivities in ranked order (D_1 , D_2 , and D_3), fractional anisotropy (FA), and the volume fraction (VF) percentages are reported for both fast and slow diffusion tensors. Asterisks (*) denote $P < 0.05$; diffusivity units ($\times 10^{-3}$ mm²/s).

Slow	D130c (N=5)	5mc (N=5)
D_1	0.38 $\pm$ 0.03	0.46 $\pm$ 0.03
D_2	0.11 $\pm$ 0.02*	0.20 $\pm$ 0.02
D_3	0.07 $\pm$ 0.01*	0.12 $\pm$ 0.02
FA	0.72 $\pm$ 0.02*	0.59 $\pm$ 0.02
VF%	37 $\pm$ 5*	21 $\pm$ 2

Fast	D130c (N=5)	5mc (N=5)
D_1	1.15 $\pm$ 0.05	1.07 $\pm$ 0.03
D_2	0.50 $\pm$ 0.04*	0.70 $\pm$ 0.03
D_3	0.35 $\pm$ 0.03*	0.57 $\pm$ 0.03
FA	0.57 $\pm$ 0.03*	0.34 $\pm$ 0.01

Table 4

Quantitative confocal microscopy measurements including nucleus density (ND), myocyte length, and width, obtained from randomly selected hearts at preterm 100- (D100), 130- (D130), 140-days gestation (D140), and full term (D15), and postnatal 5-months of age (5m). Also included Lv free-wall thickness (WT, from MRI), and the ratio of wall thickness to myocyte width (MW) (indicative of hyperplasia). In each heart, 3 transmural sections (subendocardium, mid-wall, and subepicardium) were analyzed and reported as mean $\pm$ SEM.

	D100	D130	D140	D150	5m
ND (/mm ²)	7735 $\pm$ 1220	5890 $\pm$ 321	5794 $\pm$ 550	4761 $\pm$ 479	1750 $\pm$ 176
Myocyte Length (μ m)	32 $\pm$ 3	46 $\pm$ 5	47 $\pm$ 4	77 $\pm$ 3	105 $\pm$ 7
Myocyte Width (μ m)	4.6 $\pm$ 0.2	6.3 $\pm$ 0.5	6.7 $\pm$ 0.6	7.4 $\pm$ 0.7	18.5 $\pm$ 2.1
Wall Thickness (mm)	3.6 $\pm$ 0.1	5.3 $\pm$ 0.4	5.1 $\pm$ 0.1	5.3 $\pm$ 0.3	9.0 $\pm$ 0.3
WT/MW	769	848	765	714	484