Early transverse tubule development begins *in utero* in the sheep heart

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Abstract:

The ventricular cardiomyocytes of adult mammals contain invaginations of the plasma membrane known as transverse (t)-tubules. These regular structures are essential for the synchronisation of excitation-contraction (EC) coupling throughout the cell, which is a vital process for cardiac function. T-tubules form a close association with the sarcoplasmic reticulum (SR) to form junctions, where several key proteins involved in EC coupling are localised, including the SR calcium release channels – the ryanodine receptors (RyR). The lipophilic SR protein junctophilin-2 (JPH2) has been implicated in the development of both the junctions and t-tubules. Several studies have identified that t-tubules develop only postnatally in rodents, while historical EM data indicate that this is not the case in larger mammals, including humans. We have performed, to our knowledge, the first fluorescent, target-specific study to characterise t-tubule development in the large mammalian fetal heart, focussing on the sheep. T-tubules were present in fetal sheep hearts from 114 days gestation (with term being 145 days), with occurrence progressively increasing with gestational age, and further maturation after birth. This was accompanied by an increasing intracellular localisation of JPH2, which progressively increased its association with RyR within the cardiomyocytes as they undergo hypertrophy. These findings indicate that large

mammalian hearts exhibit a significantly different temporal pattern of development compared to that of the rodent. Our findings have potential implications for human cardiac development, including the future investigation of congenital heart disease.

Key words:

T-tubules; Cardiac development; Ryanodine receptor; Junctophilin-2; Excitation-contraction coupling

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Introduction

Mammalian ventricular cardiomyocytes contain plasma membrane invaginations, forming the transverse (t)-tubules, which are key structures in allowing the rapid propagation of action potentials towards the cell interior (Brette and Orchard, 2003). The disorganisation or loss of t-tubules has been widely associated with heart failure through the inability to synchronise calcium release across the myocyte (Louch et al., 2004, Sacconi et al., 2012). Despite the need for synchronised cardiomyocyte contraction during *in utero* development, in rodents, t-tubules are identified only after birth (Chen et al., 2013, Haddock et al., 1999, Sedarat et al., 2000, Ziman et al., 2010). This has been suggested as being due to differences in excitation-contraction (EC) coupling mechanisms compared to the adult heart (Louch et al., 2015). The lipophilic protein junctophilin-2 (JPH2) plays a role in the formation and maintenance of junctions, which are formed by the close association of the t-tubules and the intracellular calcium store, the sarcoplasmic reticulum (SR) (Takeshima et al., 2000). Ryanodine receptors (RyRs) form clusters within the junctional SR in close proximity to the t-tubules (Soeller et al., 2007), where they are responsible for calcium release from the SR, which is required for contraction of the cardiomyocyte.

RyRs are organised into transverse rows of intracellular clusters prior to birth in the rodent heart, where they form immature calcium release units (CRUs), reported to be aligned with the z-disks (Sedarat et al., 2000, Ziman et al., 2010, Haddock et al., 1999). In contrast, the sodium-calcium-exchanger (NCX) is confined to the surface membrane until a postnatal age at which t-tubule development is observed (Haddock et al., 1999). Expression of JPH2 is detected as early as E9.5 in embryonic mice, the age at which JPH2-knockout becomes embryonically lethal (Takeshima et al., 2000). Localisation of JPH2 is initially on the surface sarcolemma (Ziman et al., 2010), and although the exact mechanism through which t-tubules form remains unclear, JPH2 has been implicated in the development and maintenance of t-tubule organisation, with the expression level and intracellular localisation of JPH2 increasing *prior* to the onset of t-tubule formation (Chen et al., 2013, Ziman et al.,

2010). Furthermore, JPH2-knockdown results in not only delayed t-tubule development, but also disorganised t-tubules forming in postnatal mouse cardiomyocytes (Chen et al., 2013). To date, the vast majority of studies examining t-tubule development focus on small rodent models, which have shown that t-tubules can be identified only postnatally. However, some studies from previous decades suggest that this may not be the case in larger mammals. Electron microscopy (EM) work on the developing human or sheep heart indicates possible rudimentary t-tubule development in prenatal animals (Brook et al., 1983, Sheldon et al., 1976, Kim et al., 1992). Due to the nature of EM, the interpretation and extrapolation of findings on such small regions of cell membrane can be difficult, particularly in the absence of specific membrane labelling and 3D information. As such, by utilising specifically targeted fluorescent markers throughout the myocardium to accurately identify whole cell and tissue-wide membrane distribution patterns, the present study is, to our knowledge, the first detailed examination of t-tubule development and junctional protein association in a prenatal large mammal – the fetal sheep.

Materials and Methods

Sample collection and preparation

Romney/Suffolk sheep hearts were provided by the Fetal Physiology and Neuroscience Laboratory, University of Auckland, from fetal sheep of 108, 114, 125-126 and 135-136 days gestational age (GA), with term being 145 days (Davidson et al., 2015). Maternal sheep hearts were also collected and processed together with the fetal samples. All experimental animal protocols were approved by The University of Auckland Animal Ethics Committee, with animals euthanized with a lethal dose of sodium pentobarbital (150 mg/kg, i.v.). Left ventricular (LV) tissue samples were collected and immersion fixed in 1% paraformaldehyde on ice for 2 h, cryoprotected and frozen. 16 μm tissue sections were cut on a Leica 3050 cryostat and collected for immunohistochemistry, as previously detailed (Jayasinghe et al., 2012b). Sections were dual labelled with custom polyclonal rabbit anti-JPH2 (1:100; with

Western blots previously performed (Van Oort et al., 2011, Munro et al., 2016, Reynolds et al., 2016) and used for cardiac immunofluorescence (Jayasinghe et al., 2012a, Hou et al., 2015)) and either mouse anti-RyR (1:100; MA3-916, Thermo) or anti-NCX1 (1:200; R3F1, Swant) overnight at 4°C. Samples were then incubated with goat-raised species-specific secondary antibodies conjugated to Alexa Fluor 488 or 568 for 2 h at room temperature. To stain the t-tubule network, wheat germ agglutinin (WGA; 1:200) conjugated to Alexa Fluor 647 was applied concomitantly to the secondary antibodies, which is a widely used method for staining t-tubules (Crossman et al., 2015, Richards et al., 2011). Additional samples were stained with WGA (conjugated to Alexa Fluor 594) and phalloidin (Alexa Fluor 488) (1:50) to label the contractile machinery through f-actin specific binding. All secondary antibodies and stains were purchased from Life Technologies. Tissue sections were mounted onto slides with ProLong Gold (Life Technologies) and imaged using an inverted Zeiss LSM 710 confocal microscope.

Image processing and analyses

Confocal stacks of junctional protein labelling were acquired in both longitudinal and transverse orientations, and deconvolved using Richardson-Lucy maximal likelihood algorithms. To assess the density of transverse elements (clear transverse extensions of the t-system in longitudinal myocytes) of the developing t-tubule network, maximum intensity z-projections of three consecutive longitudinal WGA image slices were generated. Manual thresholding was applied to ensure the resulting mask was representative of the original image. The intracellular regions were selected and the percentage of WGA-positive pixels clearly showing transverse organisation was determined. For t-tubule prevalence assessment, single images of t-tubule labelling in the transverse orientation at the level of the z-disk were analysed with percentile thresholding applied, such that 0.5 signal fraction is attributed to background labelling. Intracellular regions were selected, with the percentage of WGA-positive pixels determined. Average cardiomyocyte diameter was calculated from manually measuring the cell perimeter within transverse sections.

To determine the development of junctional organisation, the co-localisation of RyR and JPH2 was assessed in longitudinal images of the dual labelled samples. Using ImageJ, isodata thresholding was applied to each protein label, with the surface membrane selectively excluded to generate a binary mask of intracellular labelling only. The two protein masks were then overlaid, with the number of co-localised pixels determined and converted into the percentage of total pixels. Changes in both protein expression and t-tubule prevalence prevented a consistent threshold for co-localisation with t-tubule labelling being determined with confidence.

Statistical analysis

Unless otherwise stated, data is presented as mean ± standard error of the mean (SEM). One-way ANOVA with post-hoc comparisons was performed on the data using IBM SPSS Statistics v22 software, with $p<0.05$ considered significant. All n-numbers are reported in corresponding figures legends.

Results and Discussion

T-tubule development in the fetal sheep heart

Confocal imaging revealed visible striations along the myofibrils, visualised by phalloidin staining of f-actin, in the youngest sheep hearts examined (108 days GA; Fig 1A). These were maintained throughout all ages of development, indicating myofibrils were organised into sarcomeres, including z-disk formation. Using the sarcolemmal marker WGA, there was some evidence of tubular membrane extensions present in the 108 day GA hearts, predominantly in the transverse view (Fig 1C), with occasional small membrane buddings identified in some cardiomyocytes. As development progressed, there was increasing evidence of plasma membrane invaginations into the cardiomyocytes that may represent the beginning of widespread t-tubule formation in the fetal sheep heart; these invaginations were typically aligned with the z-disks. This was observed by the overall increased striated

appearance of WGA staining in longitudinal views of cardiomyocytes (Fig 1, middle panels) as well as a greater number of sarcolemmal internal buddings in transverse cross-sections through cells (Fig 1, right-hand panels). By 135-136 days GA, there were clear (likely still immature compared to adult structure) t-tubules identified within the majority of cardiomyocytes, with moderate lengths of the membrane observed extending toward the cell interior (Fig 1K,L). These were observed as multiple invaginations occurring at neighbouring z-disks (as determined by phalloidin staining) within single cardiomyocytes, indicating that t-tubule development begins *prior to birth* in the sheep heart. In the adult myocardium, WGA staining showed, as expected, the presence of regular t-tubules along the entire length of the cardiomyocyte which also aligned with the z-disks (Fig 1N). In all adult transverse cardiomyocytes, a clear radial spoke-like arrangement was observed (Fig 1O), as previously identified in the adult sheep ventricle (Richards et al., 2011) and similar to the reported adult human phenotype (Jayasinghe et al., 2012b).

Quantitative analysis confirmed the visual observations of a low occurrence of t-tubules in younger animals which increased with developmental age. Transverse element density progressively increased (Fig 2A), with 125-126 days GA significantly increased compared to 108 day GA sheep, and further maturation observed in the adult heart compared to the oldest fetal heart (Fig 2A). Assessment of transverse cardiomyocytes at the level of the z-disk revealed that with increasing GA, there was a continual and significant increase in the prevalence of t-tubules compared to the most immature fetal cardiomyocytes at 108 days GA (Fig 2B). Compared to the 135-136 day GA fetus, this t-tubule development was also found to be further increased in the adult heart. Together, these findings demonstrate a progressive development of the t-tubule system which begins *in utero* in the sheep heart, with further maturation occurring after birth towards adulthood. This is in support of previous qualitative EM work, which suggests early membrane invaginations are present in the fetal sheep from 90 days GA, and t-tubules from 110 days GA onwards (Sheldon et al., 1976, Brook et al., 1983). In contrast to the earlier EM work, the importance of the present study was the use of immunofluorescence, membrane targeted staining and quantitative analysis.

This greatly facilitates the interpretation of structures and identification of tubular invaginations. By staining with WGA and imaging relatively large myocardial sections, there is a greatly improved ability to identify the presence and pattern of t-tubule distribution throughout the entire cardiomyocyte. This is compared to previous EM approaches which rely on partial sections of myocardium to interpret ultrastructural information on a greater scale, typically without clear indication of tissue orientation or verified identification of membrane structures. The inclusion of transverse sections in the present study also enables the clear identification of the pattern of t-tubule extensions throughout the cardiomyocyte cross-section, which to our knowledge has not been previously examined in the developing large mammal.

It was also evident from transverse myocardium sections that cardiomyocyte diameter increased with gestational age (Fig 1, right-hand panels). Cardiomyocyte diameter was significantly increased from 126 days GA onwards compared to the 108 GA samples, with adult cardiomyocytes clearly the largest (Fig 2C). The progressive enlargement of the cardiomyocytes was aligned with the increasing appearance of t-tubules in the fetal sheep heart, supporting the idea that as cardiomyocytes develop and undergo hypertrophy, there is a subsequent increased need for t-tubules to maintain synchronous signalling throughout larger cells. As mammalian t-tubules are essential for enabling rapid and synchronised EC coupling throughout the cell (Brette and Orchard, 2003), the requirement for t-tubules becomes more critical in thicker myocytes to prevent a substantial delay in intracellular EC coupling compared to sole reliance of calcium diffusion from the cell surface (Haddock et al., 1999).

Progressive junctional organisation in fetal sheep cardiomyocytes

The establishment of intracellular CRUs is important for the maturation of EC coupling processes in the mammalian heart. In order for these to form functional internal junctions, there must be an association between these SR regions and the t-tubule membrane, which has been shown to involve JPH2 (Takeshima et al., 2000). To our knowledge, there have

been no studies to date examining the distribution of EC coupling proteins in the large mammalian fetal heart. Therefore, triple labelling of RyR, JPH2 and the sarcolemma (including t-tubules) was examined in the developing sheep heart. Confocal imaging confirmed the intracellular localisation of RyR clusters in hearts of all ages, with surface labelling also evident in the 108 day GA samples (Fig 3A), in agreement with reports from neonatal rodent hearts (Sedarat et al., 2000, Haddock et al., 1999). There was an enhanced striated appearance of the RyR clusters with advancing age (Fig 3A-D), with this organisation being the most pronounced in the adult sheep (Fig 3E). In contrast, JPH2 was identified as primarily distributed along the surface sarcolemma in the younger fetal sheep, with little evidence of intracellular localisation in the 108 day GA (Fig 3A). From 114 days GA onwards, diffuse clusters of JPH2 were seen as occurring within the cardiomyocytes (Fig 3B-D). Both RyR and JPH2 clusters were often observed as associating with the developing t-tubule system in the 114-136 day GA fetal sheep hearts. In the adult sheep cardiomyocytes, RyR and JPH2 clusters were predominantly observed in regular, intracellular clusters which aligned with the mature t-tubules (Fig 3E). Analysis of RyR-JPH2 dual confocal images revealed a progressive increase in the intracellular co-localisation of these two proteins with increasing gestational age (Fig 3F). This co-localisation was significantly greater in 125-126 days GA and adult sheep, compared to the 108 day fetal sheep. The overall pattern of junctional development is paralleled by that observed in neonatal rodents, however with a clear temporal shift towards *in utero* initiation in sheep.

Changes in NCX distribution in fetal sheep cardiomyocytes

The localisation and functional role of NCX in EC coupling has been shown to dramatically change in the maturing heart (Louch et al., 2015). Therefore, this protein was also examined in the fetal sheep heart in relation to t-tubule development. In all fetal sheep myocardium samples, NCX was primarily localised to the surface sarcolemma (Fig 4A-D). In the more mature fetal sheep in which early t-tubule development was clearly observed, there was also intracellular distribution of NCX identified. In the 114 day GA hearts, NCX was aligned with

the early membrane buddings (Fig 4B), while in the 125-136 day samples, NCX was clearly associated with the immature t-tubules (Fig 4C,D). In the adult sheep myocardium, there was even further pronounced intracellular NCX labelling observed, which showed a clear striated appearance associated with the position of the t-tubules (Fig 4E). The early developing heart relies on calcium diffusion from the surface sarcolemma to initiate cellular contraction, which is at least partially provided by the reverse mode of NCX (Louch et al., 2015). With myocyte hypertrophy, this diffusion becomes inadequate to provide a synchronous signal across the cell, and so the need for t-tubules arises. As NCX becomes intracellularly distributed along the premature t-tubules, the forward-mode becomes dominant for calcium extrusion, and EC coupling shifts towards the adult phenotype of relying on L-type calcium channels for calcium influx (Louch et al., 2015).

The distribution of t-tubules in the adult human heart more closely resembles that observed in the adult sheep compared to in rodents (Jayasinghe et al., 2012b), suggesting that these findings from a large mammalian model provide greater translational implications towards human cardiac development. While the exact mechanism by which t-tubules form is currently unclear (Di Maio et al., 2007), our findings clearly demonstrate that the development of these key structures in the large mammalian heart is temporally different from that in rodents. The presence of t-tubules in the fetal sheep heart highlights the extent of developmental hypertrophy that is occurring at this stage, increasing the need for t-tubules as diffusion from the surface membrane becomes inadequate to meet cellular requirements. Combined with the increasing organisation of junctional proteins identified in the fetal sheep heart, there is also likely to be maturation of EC coupling processes prior to birth. Considering similarities between adults, this suggests the same developmental process is potentially also occurring in the fetal human heart. Identifying temporal differences in these processes is essential for understanding normal cardiac development, and how this may differ between species. There is strong evidence for the translational potential of these findings into human development, including the future investigation of pathological development, such as congenital cardiomyopathies.

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Figure Legends

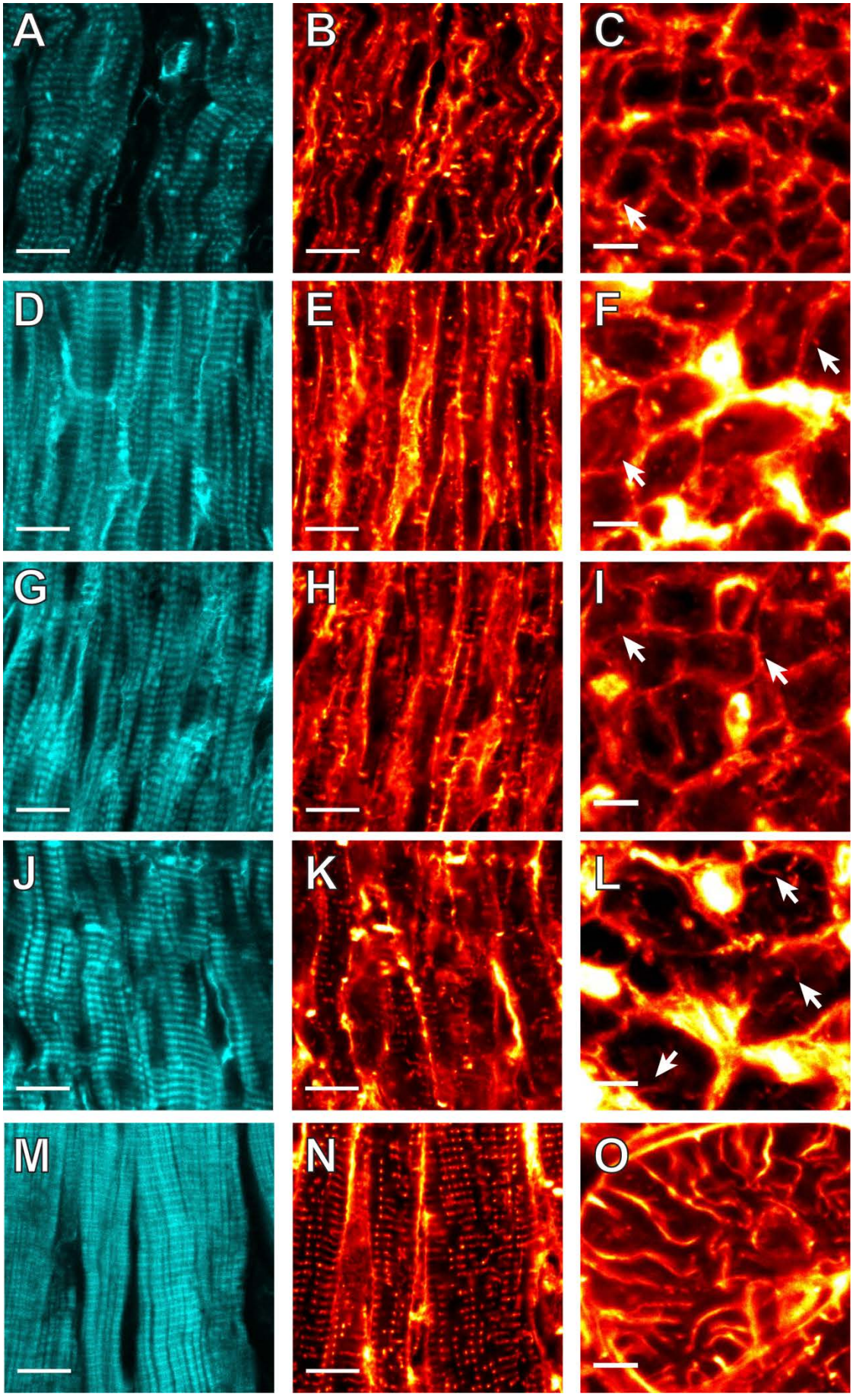
Fig 1 Evidence of t-tubule development in the fetal sheep heart. Confocal micrographs of dual f-actin (phalloidin; cyan) and t-tubule (WGA; red) labelling in LV myocardium from fetal sheep of increasing GA and adult sheep. Left-hand column displays longitudinal f-actin labelling with corresponding t-tubule labelling in middle column. Right-hand column shows t-tubule labelling in representative transverse myocardium sections. Ages: A-C) 108 days GA, D-F) 114 days GA, G-I) 125-126 days GA, J-L) 135-136 days GA and M-O) adult. Arrows indicate immature t-tubules budding from the surface sarcolemma. Scale bars: left and middle panels 10 μm, right panels 5 μm

Fig 2 Increasing t-tubule development with hypertrophy. Image analysis results for A) transverse element (TE) density in longitudinal sections, B) t-tubule prevalence in transverse section and C) cardiomyocyte diameter in the developing fetal and adult sheep myocardium.

Data presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared to 108 day GA sheep; ##p<0.01, ###p<0.001 adult compared to 136 day GA sheep. N-numbers as #cells(#animals): TE density: 108 GA 5(2), 114 GA 6(3), 126 GA 6(3), 136 GA 8(3), Adult 5(2); t-tubule prevalence: 108 GA 10(1), 114 GA 20(2), 126 GA 20(2), 136 GA 20(2), Adult 20(2); diameter analysis 108 GA 50(1), 114 GA 110(2), 126 GA 110(2), 136 GA 100(2), Adult 43(2)

Fig 3 Development of junctional protein organisation. Deconvolved confocal micrographs of RyR (red) and JPH2 (green) dual labelling in myocardium, with corresponding t-tubule (WGA; grey) staining in longitudinal sections from A) 108 day, B) 114 day, C) 125-126 day, D) 135-136 day gestation fetal and E) adult sheep hearts. Arrowheads indicate example regions of RyR-JPH2 association aligning with developing t-tubules. Scale bars: 5 μm. F) Resulting image analysis of RyR-JPH2 association in the developing sheep myocardium. Data presented as mean ± SEM. **p<0.01 compared to 108 day GA sheep. N-numbers as #cells(#animals): 108 GA 6(3), 114 GA 7(3), 126 GA 4(2), 136 GA 8(3), Adult 5(2)

Fig 4 NCX organisation with developing t-tubules. Deconvolved confocal images of NCX (magenta) and corresponding t-tubule (WGA; grey) staining in longitudinal sections from A) 108 day, B) 114 day, C) 125-126 day, D) 135-136 day gestation fetal and E) adult sheep hearts showing an increasing intracellular distribution. Scale bars: 5 μm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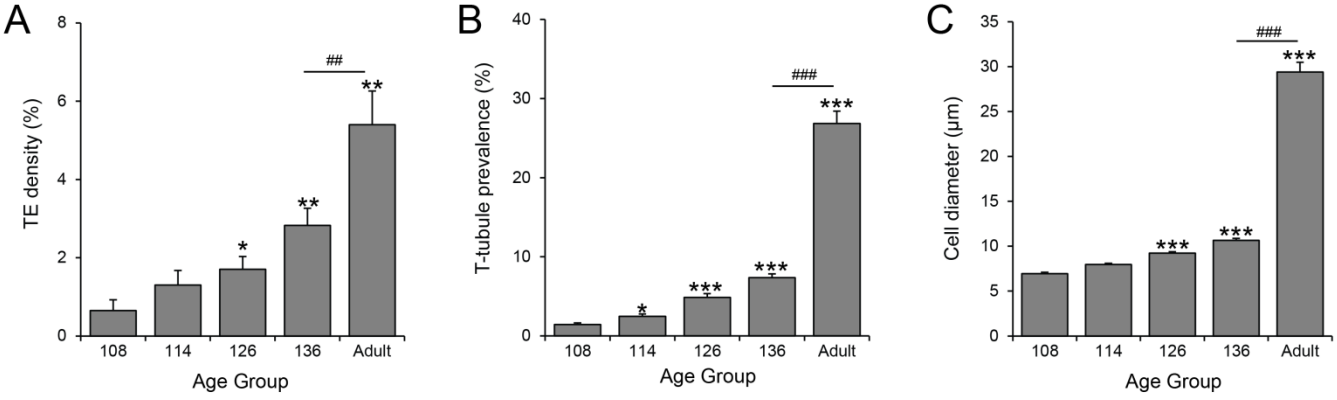
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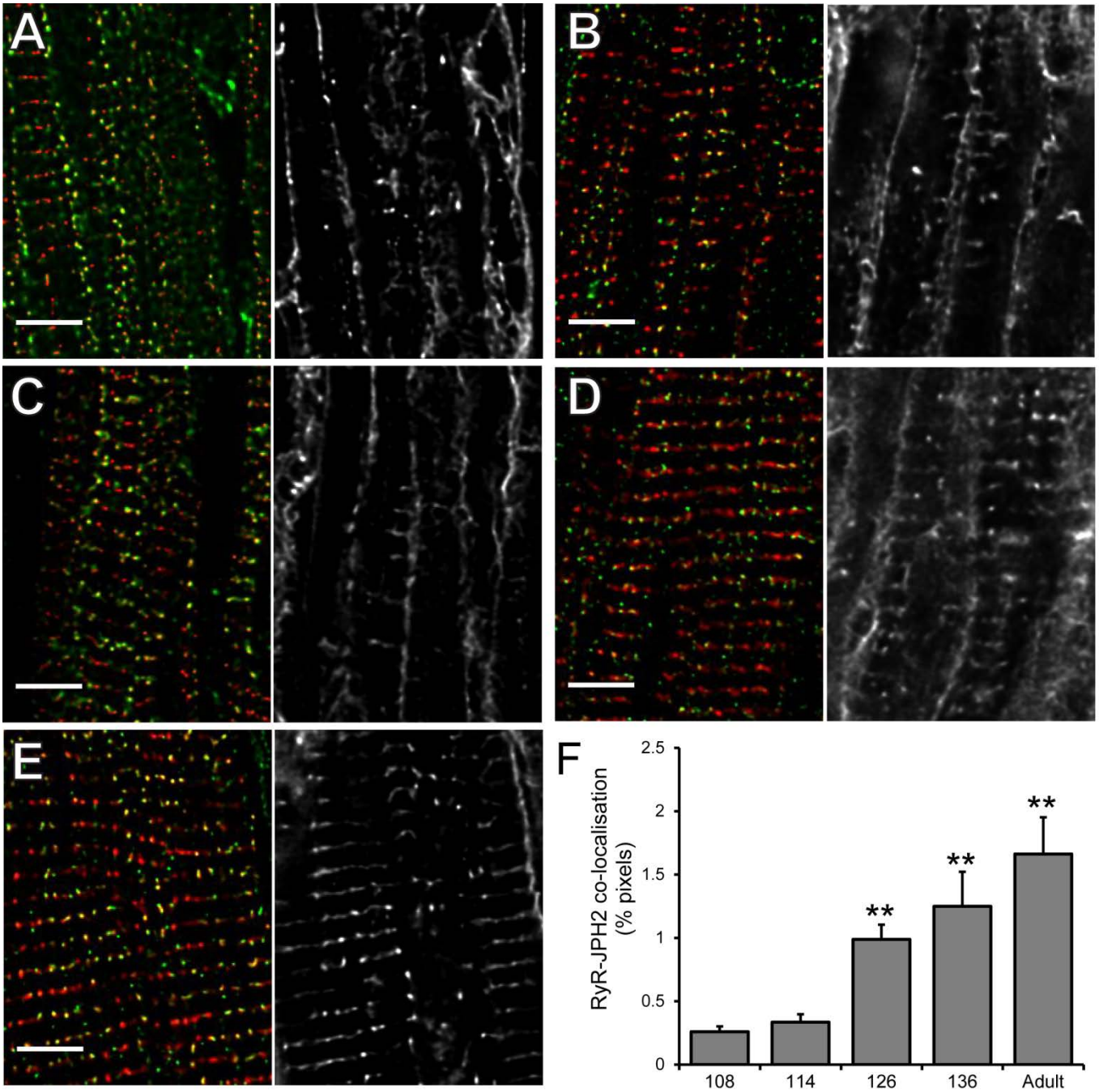
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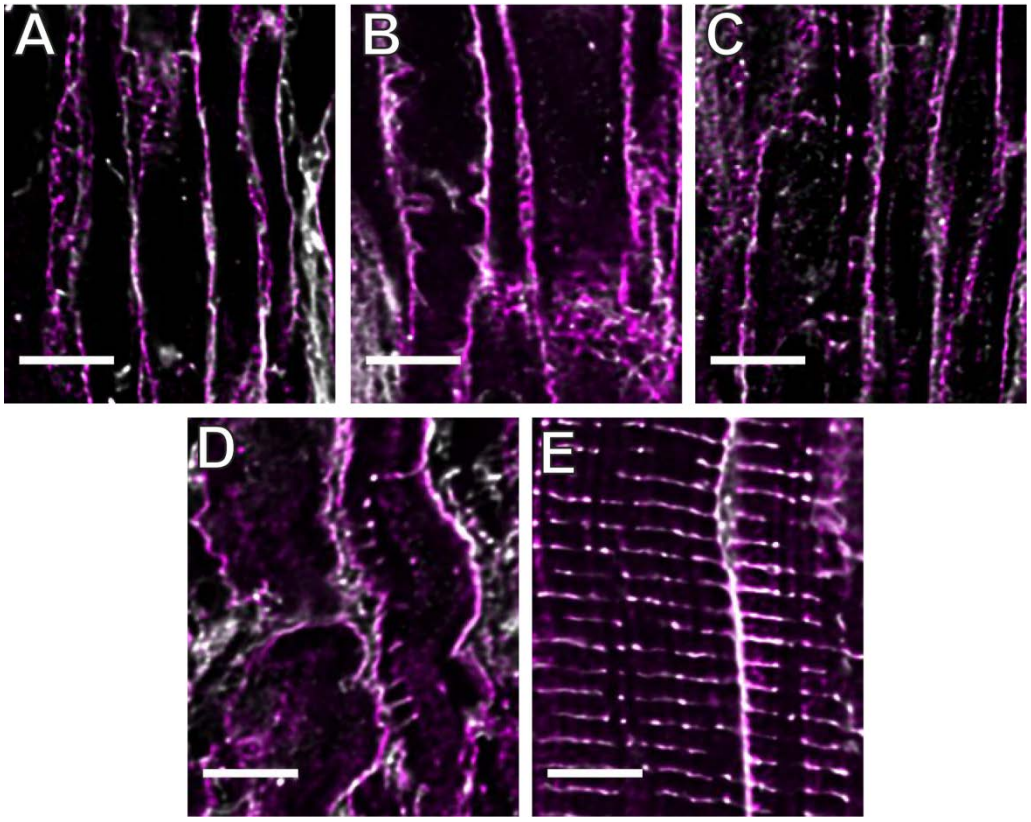
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