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Plasticity of cardiac titin/connectin in heart development☆

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Abstract

Many sarcomeric proteins in the myocardium alter their isoform pattern during perinatal development to adjust to the intensified pump function of the postnatal heart. These changes also involve the giant protein titin/connectin. Here we show by low-percentage polyacrylamide-gel electrophoresis that developmentally regulated switching of cardiac titin/connectin size occurs in the hearts of mouse, rat, pig, and chicken. Mammalian hearts express, well before birth, large foetal (~3.7 MDa) N2BA-titin/connectin isoform but no N2B-isoform (3.0 MDa). During perinatal heart development the 3.7-MDa N2BA-isoform is replaced by a mix of smaller isoforms. At birth a plethora of intermediate-size N2BA-isoforms appears together with the N2B-isoform. In postnatal heart development the larger-size N2BA-isoforms disappear and smaller-size N2BA-isoforms are upregulated, whereas the proportion of N2B-titin/connectin increases to species-specific adult levels. The time courses of isoform switching are faster in small than in large mammals. Titin/connectin isoform switching also takes place in developing chicken hearts, but the largest embryonic isoform found here was less than 3.4 MDa. At hatching, various smaller-size isoforms appeared and within a week the adult expression pattern was established representing a major 3.0-MDa isoform and a minor 3.15-MDa isoform. The ratio between the two adult isoforms differed between the left ventricle and the right atrium. The perinatal changes toward smaller cardiac titin/connectin isoforms in mammals and chicken greatly increase the myofibrillar passive tension of postnatal hearts. Plasticity of titin/connectin at approximately the time of birth thus affects myocardial mechanics but could also be an important factor in developmentally regulated assembly and signalling processes.

Introduction

In a human fetus the heart starts to beat at gestational day 22 and from then on does not stop sometimes for even 100 years. Of course a dramatic event in mammalian heart development is birth. With the first breaths of air the newborn takes, the foetal circulation changes and myocardial pump function must be intensified quickly to keep up with the increased power requirements of the newborn that suddenly lacks placental nurturing and oxygen supply. At birth the foetal heart also experiences great changes in mechanical constraints. *In utero*, extracardiac constraints are imposed by the liquid-filled lungs, the pericardium and a restricted space for thoracic movements, which together limit the extensibility of the foetal heart (Grant *et al.*, 1992, 2001). With birth and with the aeration of the lungs, the extracardiac constraints are substantially reduced (Grant and Walker, 1996). Heart rate, left ventricular (LV) dimensions, end-diastolic pressure, and stroke volume all increase to meet the metabolic demands of newborn life (Kirkpatrick *et al.*, 1973, Anderson *et al.*, 1982). As predicted by the law of Laplace, the enlargement of the LV cavity elevates the levels of LV wall stress in the neonate. Although the pericardium poses limits to the overall extensibility of the ventricles, the reduction in extracardiac constraints at birth may require the postnatal heart to protect itself from damaging mechanical forces by increasing its own mechanical strength and stiffness.

Myocardium responds to this situation with relatively rapid changes in protein expression patterns. At the myofibril level, many proteins have been shown to switch their isoform type at approximately the time of birth. For instance, in rat ventricle the β-myosin heavy chain (β-MHC), the predominant foetal isoform, is largely replaced during early postnatal development by α-MHC (Hoh *et al.*, 1978; Lompré *et al.*, 1984; Capelli *et al.*, 1989) and the atrial/embryonic myosin light chain-1 present in foetal ventricles disappears after birth (Whalen and Sell, 1980). Similarly, both the cardiac (60–70%) and the skeletal (30–40%) α-actin isoforms are expressed before birth, whereas mainly the cardiac α-actin (95%) is found in the adult rat heart (Carrier *et al.*, 1992). Many other proteins, including troponin-I, troponin-T, tropomyosin, and myomesin, switch isoforms shortly after birth

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(Saggin *et al*., 1988, 1989, Reiser *et al*., 1994; Agarkova *et al*., 2000;Metzger *et al*., 2003;). Developmental changes in myocardial contractile and regulatory protein expression have also been described for many other mammalian species (Fisher and Towbin, 1988; Lyons *et al*., 1990; Murphy, 1996; Morimoto and Goto, 2000; Siedner *et al*., 2003) and for chicken hearts (Lim *et al*., 1983; Sabry and Dhoot, 1989; Godt *et al*., 1993 In perinatal heart development essentially most proteins involved in active force generation and various proteins involved in myofibrillar force transmission undergo isoform switching.

We (Opitz *et al*., 2004) and others (Lahmers *et al*., 2004; Warren *et al*., 2004) have shown recently that also the giant sarcomeric protein connectin (Maruyama *et al*., 1976; 1977a, b), which is often called titin (Wang *et al*., 1979, 1984; Trinick *et al*., 1984), alters its cardiac-isoform pattern at approximately the time of birth. Titin/connectin, the largest protein known to date (3-4 MDa; Labeit and Kolmerer, 1995; Tskhovrebova and Trinick, 2003), provides the sarcomere with a structural framework through association with many other myofibrillar, cytoskeletal, and cytosolic proteins (Miller *et al*., 2004). The >1-μm-long molecules span half-sarcomeres (Fürst *et al*., 1988; Itoh *et al*., 1988) and help centre the A-band in the sarcomere during and after active contraction (Horowits *et al*., 1986). The I-band portion of titin/connectin (Figure 1) determines passive-tension development and elasticity of cardiac myofibrils (Funatsu *et al*., 1993; Linke *et al*., 1994, 1999; Trombitas *et al*., 1995; Kulke *et al*., 2001; Granzier and Labeit, 2004), acts as a rapidly recoiling spring during muscle shortening (Opitz *et al*., 2003), and may have a role in the Frank-Starling mechanism of the heart (Cazorla *et al*., 2001; Fukuda *et al*., 2001, 2003). The functionally elastic cardiac titin/connectin segment consists of three structurally distinct segments (Labeit and Kolmerer, 1995, Linke *et al*., 1999): Ig-domain regions (red colour in Figure 1), the PEVK-domain (yellow colour), and a "unique N2B-sequence" (blue colour in Figure 1). Upon muscle stretching from slack sarcomere length (SL), the Ig-domain regions extend first, presumably by straightening interdomain linkers (Gautel *et al*., 1996; Linke *et al*., 1996). Modest but still physiological forces are needed to extend both the PEVK-domain and the unique N2B-insertion (Linke *et al*., 1998, 1999; Trombitas *et al*., 1999; Linke and Fernandez, 2002). Unfolding of Ig-domains (Kellermayer *et al*., 1997; Rief *et al*., 1997; Tskhovrebova *et al*., 1997; Li *et al*., 2002) could take place to a very limited degree in cardiac sarcomeres (Linke and Leake, 2004), but the issue is still controversial (Minajeva *et al*., 2001; Trombitas *et al*., 2003). In mammalian myocardium, two principal isoforms of titin/connectin, the so-called "N2B" and "N2BA" isoforms (Figure 1), are generated by alternative splicing of the transcript of a single gene of titin/connectin (Labeit and Kolmerer, 1995; Freiburg *et al*., 2000; Greaser *et al*., 2002). The differentially spliced segments encompass the "mid-Ig region" and the PEVK domain (Figure 1). In addition, a so-called N2-A segment is present in all N2BA-isoforms, but not in the N2B-isoform. The N2B and N2BA isoforms are incorporated both into the same sarcomere (Linke *et al*., 1996; Trombitas *et al*., 2001; Neagoe *et al*., 2002). The proportions of these isoforms are now known to be variable in the heart under both physiological and pathological conditions.

Expression of cardiac titin/connectin isoforms varies from predominantly N2B in small adult rodents, such

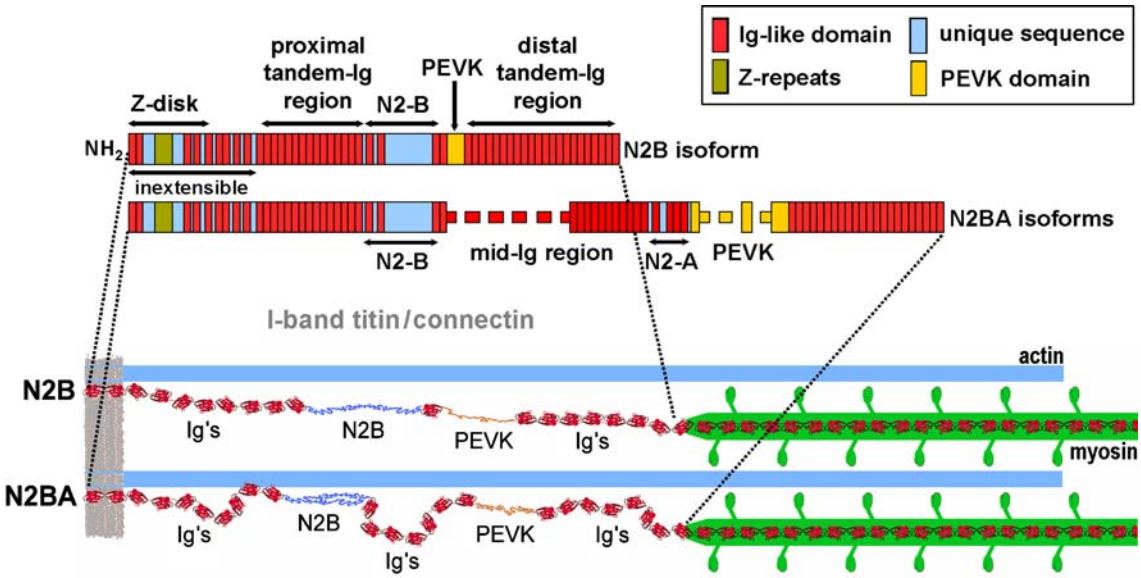

Fig. 1. Domain structure of I-band titin/connectin isoforms in a cardiac sarcomere according to Freiburg *et al.* (2000). The elastic region of the molecule encompasses three structurally distinct segments: serially linked tandem-Ig domains, the PEVK-domain, and the N2B-unique sequence. Two principal isoforms, N2B and N2BA, are co-expressed in the same half-sarcomere and may extend independently. The length differences between the two main cardiac isoforms are caused by alternative splicing of the mid-Ig domains and PEVK exons. For clarity, the sarcomere model (bottom) does not show the real number of titin/connectin domains known from sequence studies.

as rat, to predominantly N2BA in large mammals, such as cow (Cazorla *et al*., 2000; Neagoe *et al*., 2003). In normal human myocardium the N2B:N2BA-ratio is approximately 70:30 (Neagoe *et al*., 2002; Makarenko *et al*., 2004). Co-expression of titin/connectin isoforms at various ratios in the heart is an effective means for tuning titin/connectin-based passive tension. In normal adult human heart, titin/connectin-based stiffness constitutes a major proportion of total myocardial passive stiffness (Makarenko *et al*., 2004); the contribution of titin/connectin to total passive stiffness surpasses that of extracellular matrix material, including collagen. In chronic human heart disease, the titin/connectin-based contribution to total myocardial stiffness is significantly reduced and much of this effect stems from an increase in the proportion of longer, more compliant, N2BA-isoforms (Neagoe *et al*., 2002; Makarenko *et al*., 2004; Nagueh *et al*., 2004). These changes benefit myocardial diastolic function. Modifications in cardiac N2B:N2BA protein ratios have also been observed in cardiomyopathic hearts of rat (Neagoe *et al*., 2002; Warren *et al*., 2003) and dog (Wu *et al*., 2002).

During development the heart experiences even greater and more rapid alterations of dimensions and hemodynamics than during disease. Therefore it may be expected that at approximately the time of birth, the heart also needs to modify the mechanical properties of the titin/connectin spring to adjust to the global mechanical requirements. Here we have analyzed the perinatal and adult isoform-expression pattern of cardiac titin/connectin in the hearts of three mammalian species, mouse, rat, and pig, and of chicken heart. A bird species was included, because the changes in blood circulation and myocardial pump function at hatching are quite different from those taking place at birth of a mammal. We found that all mammalian hearts studied express, well before birth, large (approximately 3.7 MDa) foetal titin/connectin isoforms, but no N2B-isoform (3.0 MDa). During perinatal development the highly extensible ~3.7-MDa N2BA-isoforms are gradually replaced by smaller-size, less extensible, N2BA-isoforms and the least extensible N2B-isoform. A similar kind of titin/connectin-isoform switching was observed in chicken hearts, but even the largest embryonic isoform found here was no greater than 3.4 MDa. The perinatal titin/connectin-isoform switching may be a general property of mammalian and bird hearts, but the alterations in expression patterns occur with species-specific time courses and involve many different size classes of titin/connectin.

Materials and methods

Heart tissue

Foetal, neonatal, and adult rat (Sprague-Dawley) and laboratory mouse hearts were obtained from the university's animal house, in accordance with institutional guidelines. The hearts of neonatal and adult domesticated pigs were obtained from a local slaughterhouse. A pregnant wild boar was provided by a local hunter (we thank Anita Kühner for accessing and handling the heart tissue). The fetus of the wild boar was estimated to be at a stage approximately three weeks before birth. Chicken hens of the strain "Tetrabrown" were obtained from a local provider (Müller, Eppingen-Elsenz, Germany). Chicken eggs were hatched in an incubator at 37°C and embryonic and neonatal hearts were removed according to institutional guidelines. The explanted hearts were immediately frozen in liquid nitrogen and stored for less than 4 weeks at −80°C. In the case of the pregnant wild boar, the hearts were frozen within 1 h after death.

Sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE)

To investigate titin/connectin expression in cardiac muscles at various time points during development, frozen whole hearts or if dissectible, left ventricles, were homogenised in ice-cold salt buffer supplemented with 40 μg/ml leupeptin. For details, see Neagoe *et al*. (2002), Opitz *et al*. (2004), Makarenko *et al*. (2004). After brief centrifugation, solubilisation buffer (1% SDS, 1% 2-mercaptoethanol, 10% glycerol, 8 μg/ml leupeptin, 6 μM bromphenol blue, 4.3 mM Tris–HCl, pH 8.8, 4.3 mM EDTA) was added to the pellet, samples were incubated for 5 min on ice and then boiled for 3 min. Total protein content in solubilised samples was determined spectrophotometrically. Attempts were made to load the lanes on gels with approximately equal amounts of solubilised protein.

Agarose-strengthened SDS-PAGE (polyacrylamide concentration, 2%) was done as described (Linke *et al*., 1997; Neagoe *et al*., 2002, 2003) using a Laemmli buffer system (Tatsumi and Hattori, 1995). Protein bands were visualised with Coomassie brilliant blue R or by silver staining. Gels were digitised and densitometric measurements were performed using TotalLab software (Phoretix, Newcastle, UK). Western blotting has been employed in earlier studies from our laboratory to identify the isoform types of titin/connectin in mammalian tissues (Neagoe *et al*., 2002; Makarenko *et al*., 2004; Opitz *et al*., 2004).

Results and discussion

The aim of this study was to establish similarities and differences in cardiac titin/connectin isoform switching during perinatal heart development of mammalian species of different body size and of chicken. We report dramatic alterations in titin/connectin isoform size in developing chicken, mouse and pig hearts and compare them with those reported previously for rat myocardium (Lahmers *et al*., 2004; Opitz *et al*., 2004;

Warren *et al*., 2004). We begin by reviewing the current state of knowledge regarding isoform shifts in developing rat heart.

Titin/connectin in rat heart development

Figure 2 summarises the time course of cardiac titin/connectin isoform switching in perinatal development and the consequences for myofibrillar passive tension, based on the results of Opitz *et al*. (2004). Foetal hearts six days before birth (e16) show a single titin/connectin band at 3.7 MDa, which is of the N2BA-type (Opitz *et al*., 2004; Warren *et al*., 2004) and is termed N2BA-3700 isoform (Figure 2c). In foetal-e16 rat hearts no N2B-isoform band is detectable, as reported previously by Opitz *et al*. (2004) and Warren *et al*. (2004). There appear, however, relatively strong bands at ~2.2–2.3 MDa, which are truncated forms of titin/connectin probably resulting from protein degradation (Figure 2a–c). These "T2-titin" or "β-connectin" bands can be marked on Western blots by antibodies to A-band titin/connectin, but not by I-band titin/connectin antibodies N-terminal to the PEVK-domain (Opitz *et al*., 2004). The T2-titin/β-connectin bands thus contain the A-band segment of titin/connectin and probably the distal tandem-Ig region (Figure 1); the proteolysis site is likely to be within the PEVK-domain. It has been speculated (Opitz *et al*., 2004) that the strong T2-titin/β-connectin bands could be indicative of a high protein turnover rate.

About three days before birth (stage e19) a 3.6-MDa titin/connectin isoform (N2BA-3600) appears together with the 3.0-MDa N2B-isoform (Figure 2b). The ratio of N2BA-3700 to N2BA-3600 to N2B is approximately 70:20:10 (Figure 2c). On the day of birth this ratio switches to 20:40:40 (Figure 2c) or similar values (Lahmers *et al*., 2004; Warren *et al*., 2004). Following birth, the N2BA-3700 isoform disappears within 7-10 days (Opitz *et al*., 2004; Warren *et al*., 2004), but also N2BA-3600 decreases gradually and is undetectable three weeks after birth (~21 d), whereas N2B quickly becomes the predominant isoform of the postnatal left ventricle (Figure 2c). Between 10d and 15d, an N2BA-isoform of 3.4 MDa (N2BA-3400) appears and is soon followed by another, even smaller, adult N2BA-isoform of 3.2 MDa (N2BA-3200). The adult rat left ventricle contains less than 10% of these N2BA-isoforms, the remainder being N2B-isoform. This time course of titin/connectin isoform switching is similar to that occurring with myosin and troponin-I in rat-heart development (Warren *et al*., 2004).

The changes in titin/connectin size have great consequences for myofibrillar passive tension development (Figure 2d). There is agreement that the switch toward smaller titin/connectin isoforms in postnatal myocardium elevates the titin/connectin-based passive tension (Lahmers *et al*., 2004; Opitz *et al*., 2004; Warren *et al*., 2004). Between foetal-e16 and adult rat cardiomyofibrils the passive tension level increases at least by an order of magnitude (Figure 2D; Opitz *et al*., 2004). These changes in titin/connectin-based passive tension can be explained by a decrease in the lengths of the extensible spring elements in the sarcomeres during development: foetal and neonatal titin/

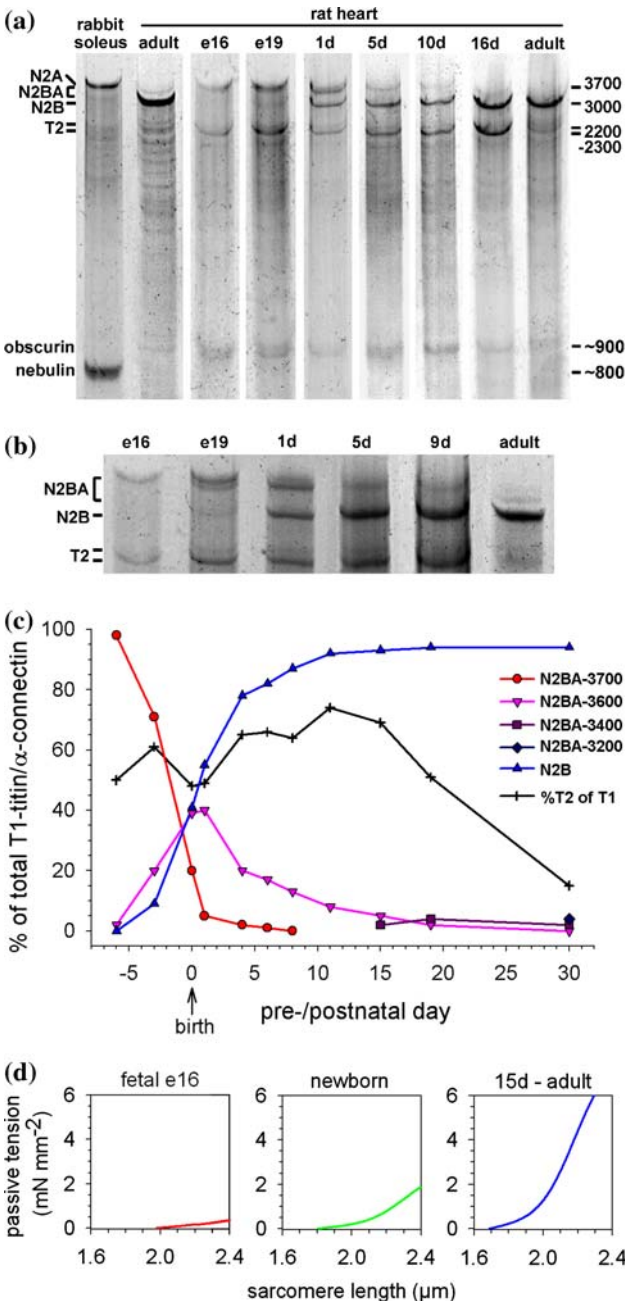

Fig. 2. Titin/connectin-isoform expression in developing rat hearts and consequences for myofibrillar passive tension. (a) 2% SDS-polyacrylamide gels showing the perinatal switching of rat cardiac isoforms. In adult rabbit-soleus tissue, titin/connectin is 3.7 MDa. (b) Higher-magnification gel image (rat left ventricles). (c) Summary scheme showing developmental changes in the composition of rat cardiac titin/connectin isoforms. Data are given in % (N2BA + N2B = 100%). "T2" indicates degradation band(s), T2-titin or β-connectin, and is shown as percentage of N2BA + N2B. (d) Differences in passive-tension development in myofibrils of foetal, neonatal and adult heart. Data were compiled from Opitz *et al*. (2004), Figures 4 + 7. e, gestational day; d, postnatal day.

connectin isoforms contain a greater number of mid-Ig domains and express more PEVK exons than adult isoforms (Lahmers *et al*., 2004; Warren *et al*., 2004). This scenario holds true for animal species as well as humans (Lahmers *et al*., 2004). In rat heart, the N2BA-3700 isoform has highly extensible mid-Ig and PEVK segments, whereas the N2BA-3600 isoform contains somewhat less extensible segments (Opitz *et al*., 2004). The least extensible isoform is N2B, which lacks the mid-Ig region and has a very short PEVK-domain. In sarcomeres with long spring elements, as in foetal heart, a given stretch results in a relatively low fractional extension (actual extension over maximum extension or contour length) and this causes low passive tension (Lahmers *et al*., 2004; Opitz *et al*., 2004). If the sarcomeres express mainly the short spring elements, as in adult rat heart, high passive tension is generated. These findings suggest that the replacement of compliant foetal titin/connectin with stiff N2B-isoform during perinatal development may be important to provide the postnatal heart with elevated passive stiffness.

Titin/connectin is not the sole determinant of passive stiffness in the heart, as the extracellular matrix (mainly collagen) also plays an important role (Linke *et al*., 1994; Granzier and Irving, 1995; Wu *et al*., 2000). In the hearts of adult rodents, including rat (Granzier and Irving, 1995) and rabbit (Linke *et al*., 1994), collagen dominates passive-tension development when sarcomeres are stretched above 2.15–2.2 μm. At shorter sarcomere lengths, collagen contributes much less than titin/connectin to the passive stiffness (Granzier and Irving, 1995). Also the contribution from intermediate filaments is minor, as they generate only $<10\%$ of the total passive tension (Granzier and Irving, 1995). Several studies have shown that interstitial collagen accumulates in developing heart immediately following birth (Borg *et al*., 1982; Carver *et al*., 1993; Engelmann, 1993) It is thus possible that titin/connectin and collagen act synergistically to increase passive stiffness in postnatal myocardium. However, the role of titin/connectin in postnatal stiffening is probably no less important than that of collagen (Lahmers *et al*., 2004). Additional studies are necessary to determine whether the rapid increase in titin/connectin-based passive stiffness at approximately the time of birth is a pre-requisite for normal mechanical function of the postnatal heart.

Mouse heart

By microarray analysis Lahmers *et al*. (2004) demonstrated that neonatal mouse hearts upregulate specifically the mid-Ig and PEVK exons when compared to left ventricles of adult mice. Newborn mouse hearts expressed both the N2BA and N2B titin/connectin isoforms, but the N2BA:N2B ratio decreased from $\sim$1.2 at birth to $\sim$0.2 in 4 d-old neonates. By using high-resolution SDS-PAGE we find that mouse hearts express, at embryonic day e14, a single large titin/connectin band of 3.7 MDa (Figure 3). At this early developmental stage essentially no N2B-isoform is detectable, whereas degradation bands (“T2”, Figure 3) are relatively strong. In 1d-old newborn mouse hearts, at least three large to mid-size titin/connectin isoforms, most probably of the N2BA-type, appear together with an already strong N2B-band (Figure 3a). On silver-stained SDS-polyacrylamide gels, the N2BA-isoforms at 1 d often appeared as a very broad band (Figure 3b) indicating the presence of multiple isoforms covering a size-range of approximately 3.3-3.7 MDa. In adult mouse heart we detected a single minor N2BA band ($\sim$3.25 MDa) and the major N2B band (3.0 MDa) at a ratio of approximately 20:80 (Figure 3), in agreement with previous findings (Neagoe *et al*., 2003).

The foetal 3700-kDa isoform expressed in e14-mouse hearts decreased already before birth and made up only $\sim$10% of all titin/connectin isoforms on the day of birth (Figure 3). A comparable prenatal reduction of the 3700-kDa isoform occurs in rat heart (Figure 2c). The postnatal time course of decrease of

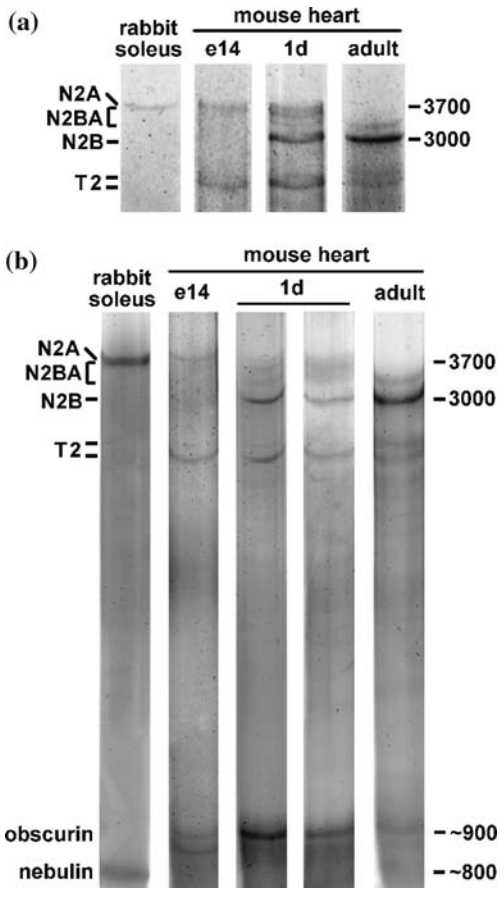

Fig. 3. Developmentally regulated switching of giant proteins in mouse myocardium analysed by 2% SDS-PAGE. (a) Coomassie-blue-stained gel showing perinatal switch of titin/connectin isoforms. (b) Silver-stained gel. Adult rabbit soleus muscle (3.7 MDa) was added for comparison of molecular weight.

N2BA isoforms was not investigated by us in mouse hearts. Elsewhere, Lahmers *et al.* (2004) have shown that the time required for a 50% reduction in N2BA titin/connectin isoforms is 2.5 days for neonatal mouse heart. Considering these findings, it appears that the postnatal titin/connectin isoform switching occurs slightly faster in mouse than in rat hearts: Figure 2c indicated that the predominant neonatal N2BA isoform in rat heart – 3600 kDa – decreases by 50% within four days, longer than the 2.5 days reported for mouse (Lahmers *et al.*, 2004). In turn, the time course for rat (4 days) is shorter than the 7 days reported for rabbit (Lahmers *et al.*, 2004). Comparing the three rodent species, the rate of early postnatal isoform switching is fastest in mouse, slower in rat and slowest in rabbit hearts.

We conclude that also mouse hearts switch from a large, 3.7-MDa, early foetal N2BA-isoform to predominantly small, 3.0-MDa N2B-isoform shortly after birth. It is likely that these changes quickly increase the titin/connectin-based passive stiffness of the neonatal mouse heart by a factor similar in magnitude to that found in perinatal rat myocardium (see Figure 2d).

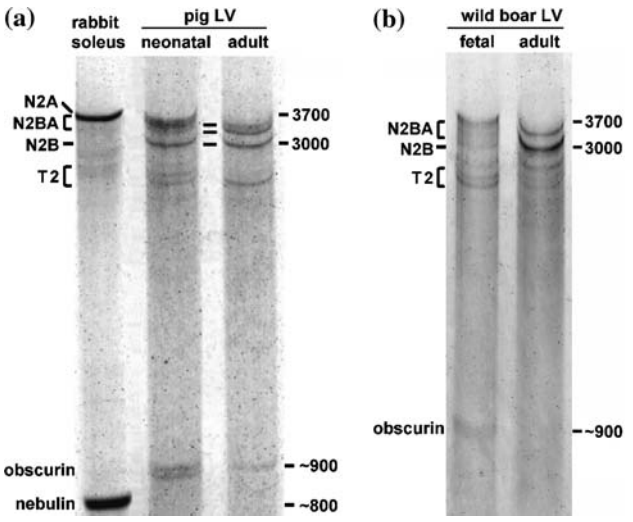

Fig. 4. Changes in titin/connectin and obscurin isoform expression in developing pig hearts (2% SDS-polyacrylamide gels). (a) Postnatal left ventricles of domesticated pigs. The neonatal heart was from a 1-2-day-old animal. Adult rabbit soleus (3.7 MDa) was added for comparison of molecular weight. (b) Foetal and adult left ventricles obtained from pregnant wild boar approximately three weeks before birth.

Obscurin

Other high-molecular-weight proteins appearing on 2% SDS-polyacrylamide gels may also undergo developmentally regulated isoform switching. One of these proteins may be the 900–1000-kDa obscurin (Figure 3b), a large modular polypeptide involved in muscle assembly and signalling processes (Young *et al.*, 2002). Elsewhere we have shown that obscurin is downregulated in adult compared to foetal/neonatal heart tissue (Opitz *et al.*, 2004). This study's results confirm and extend the earlier findings (see Figures 2a, 3b, and 4).

Pig heart

The protein composition of pigs is considered to be similar to that of humans and here we chose porcine heart to study whether cardiac titin/connectin isoforms switch from large foetal to smaller postnatal size also in large mammals (Figure 4). We obtained neonatal (1–2 day-old) and adult left ventricular tissue of domesticated pigs from a local slaughterhouse, whereas foetal (about three weeks prior to birth) and adult hearts of wild boar were provided by a local hunter. As reported previously (Lahmers *et al.*, 2004; Opitz *et al.*, 2004), neonatal pig hearts expressed a mix of intermediate-size N2BA-isoforms, which made up the majority of total titin/connectin protein, as well as less-abundant N2B-isoform (Figure 4a). In adult pig hearts N2BA migrated as a doublet band and there was still relatively more N2BA than N2B-isoform apparent. In the foetal left ventricle of wild boar we detected a strong band at ~3.7 MDa but almost no additional intact titin/connectin (Figure 4b). Adult wild boar showed a single mid-size isoform (most likely N2BA) at ~3.4 MDa and the N2B-isoform at a ratio of approximately 40:60. Degradation bands ("T2") were of similar intensities in both foetal and adult tissues (Figure 4b).

These results suggest that large mammals express, relatively early in foetal heart development, a single cardiac N2BA-isoform of 3.7 MDa, which is later replaced by smaller-size neonatal and adult N2BA-isoforms and the N2B-isoform. The time course of prenatal cardiac-isoform switching appears to be much slower in pigs than in smaller mammals (a fact probably related to the longer gestation period), as there were already various minor titin/connectin isoforms bands appearing below the major 3.7 MDa band in the foetal pig heart three weeks prior to birth (Figure 4b). Early foetal mouse and rat hearts never showed such bands. The postnatal titin-isoform switching was not studied here in pig heart, but Lahmers *et al.* (2004) found that the shift toward smaller isoforms also occurs at a much slower rate than in rodents; the time required to reduce expression of the N2BA isoforms by 50% was 18 days.

Why the adult hearts of pig and wild boar (and of other large mammals; Cazorla *et al.*, 2000; Neagoe *et al.*, 2003) express a relatively high proportion of N2BA-isoform(s) compared to those of smaller mammals, is not clear at this time. However, it is possible that large-size hearts may require lower passive wall stiffness than small-size hearts for optimum mechanical performance. The titin/connectin-isoform size would be adjusted accordingly. But no matter what

the isoform composition in adult heart, the foetal hearts of all mammals studied here express, well before birth, a very compliant N2BA-isoform of common size. This highly extensible isoform may act as a low-stiffness spring in foetal cardiac myofibrils. It will be interesting to see whether the presence of a 3.7 MDa foetal titin/connectin isoform is a general property of mammalian hearts.

Chicken heart

We wanted to know whether titin/connectin-isoform switching also takes place in embryonic/neonatal bird heart. Chicken hearts were dissected at 2-day intervals beginning at embryonic stage e12 (no titin/connectin bands were detectable by SDS-PAGE at stages e8–e10), up to several days after hatching. Embryonic hearts at stage e12 expressed a relatively broad, intermediate-size, titin/connectin band, the size and appearance of which changed little until stage e16 (Figure 5a, c). Interestingly, the embryonic isoform(s) size was always less than 3.4 MDa (Figure 5c). At day e18 a mix of even smaller-size isoforms appeared and from then on a rapid shift toward small isoforms was evident, which continued beyond hatching (Figure 5b, left two lanes; Figure 5c). Only a few days after hatching (4d), the adult expression pattern was established representing a major 3.0-MDa isoform and a minor 3.15-MDa isoform (Figure 5a–c). All developmental stages, including adult, were characterised by relatively strong "T2" degradation band(s) of comparable intensity. To verify the molecular masses, we mixed either neonatal (1d) or adult chicken-heart tissue together with rabbit soleus muscle, the latter of which expresses 3.7-MDa titin/connectin isoform, and loaded the tissue mix on the same gel lanes (Figure 5a, right three lanes). The

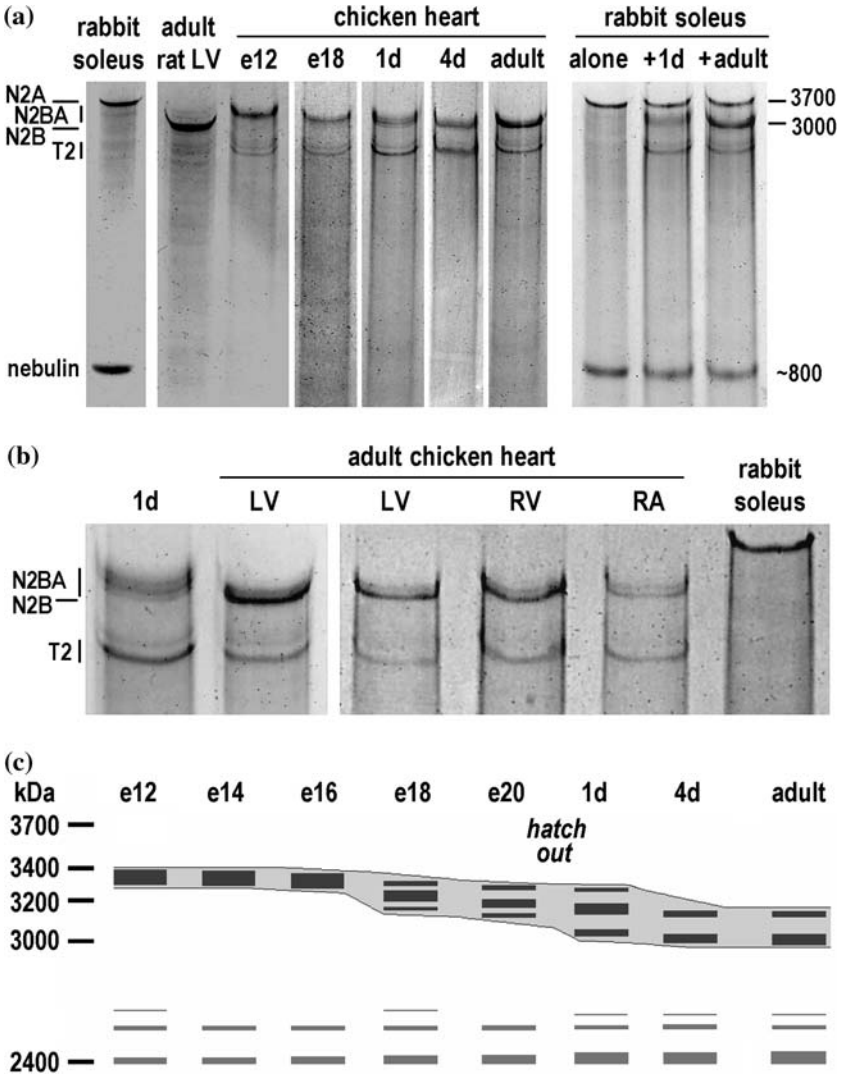

Fig. 5. Plasticity of titin/connectin in developing chicken hearts studied by 2% SDS-PAGE. (a) Time series showing isoform switching from stages e12 to adult. Adult rabbit-soleus muscle (3.7 MDa) and adult rat heart (major titin/connectin isoform, 3.0 MDa) were used to identify molecular masses. In the right two lanes, rabbit soleus was mixed together with neonatal or adult chicken-heart tissue, and the mix was charged to the same gel lanes. (b) Higher-magnification gel image showing titin/connectin bands of newborn and adult chicken hearts. LV, left ventricle; RV, right ventricle; RA, right atrium. (c) Summary scheme demonstrating the changes in isoform size and isoform composition during chicken-heart development. Grey bars on bottom indicate degradation bands ("T2").

chicken isoforms migrated faster than the soleus titin/connectin, indicating their lower molecular size. These findings establish that also chicken hearts show developmentally regulated titin/connectin isoform switching, but the largest detectable embryonic isoform is less than 3.4 MDa, much smaller than in foetal mammalian hearts. Most of the isoform shifting in perinatal chicken hearts occurs rapidly and is completed within approximately one week at around the time of hatching (Figure 5c).

In adult chicken hearts we also distinguished the left and the right ventricle and the right atrium. Both ventricles expressed a similar ratio between the two adult titin/connectin isoforms, whereas the right atrium showed a higher proportion of the lower-mobility (3.15 MDa) isoform (Figure 5B). This difference resembles that seen in adult mammalian hearts (Cazorla *et al*., 2000).

Conclusions and outlook

In this study we have provided evidence that during development from the embryonic to the neonatal to the adult stage, chicken hearts switch their cardiac titin/connectin isoform composition in a manner similar to that of mouse, rat, and pig hearts. However, the alterations in protein-expression patterns take place with species-specific time courses and involve many different titin/connectin size classes. A main difference between the bird and the mammalian species is that embryonic chicken titin/connectin never reaches the large size of the early foetal mammalian isoform (3.7 MDa), and is always smaller than 3.4 MDa. In this context we note that an earlier study reported developmental changes in titin/connectin isoforms of chicken breast muscle (Hattori *et al*., 1995), but comparisons of isoform size with the current work are difficult. Although the chicken titin/connectin sequence is not yet published, preliminary evidence (communicated by Dr. S. Labeit at the Chiba meeting, November 2004) indicates that chicken contains almost all the exons characteristic of the human titin/connectin sequence (Bang *et al*., 2001), but lacks a substantial number of PEVK exons. This raises the possibility that a much-reduced length of the chicken PEVK-domain can explain, at least in part, the relatively small size of the largest embryonic isoform in this species. It remains to be seen if the findings from mammalian heart can be extrapolated to chicken heart, in that differences in cardiac titin/connectin size between foetal, neonatal, and adult isoforms arise from alternative splicing of both the mid-Ig-domain region and the PEVK-segment.

Currently it is unknown which factors and signalling events could regulate the cardiac titin/connectin-isoform switching at approximately the time of birth. Some hints were provided by Opitz *et al*. (2004), who compared titin/connectin expression in developing rat hearts at the protein and mRNA-transcript levels. We proposed that the developmental expression pattern of the N2B-isoform may be regulated through alternative splicing, since there was a reasonable match between N2B-transcript and N2B-protein levels, at all developmental stages. As for the N2BA-isoforms, there was an excess of total N2BA-transcript over total N2BA-protein, which suggested regulation of N2BA expression at a level downstream of alternative splicing. We speculated that at least some N2BA-splice variants may have a very short half-life (mRNA stability is low), may not be translated into protein, and/or could show low translational efficiency. In any case, a challenge for future work is to find out how cardiac cells accomplish the dramatic perinatal switch of titin/connectin size by up to 700 kDa in such a short period of time. It can also be anticipated that further studies would reveal additional roles for the isoform shift, beyond the mechanical aspect. The plasticity of titin/connectin could well be an important factor in developmentally regulated muscle assembly and in myocardial signalling processes.

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