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Desmin stimulates differentiation of cardiomyocytes and up-regulation of *brachyury* and *nkx2.5*

Manuela Hofner,
Alexandra Höllrigl,
Sonja Puz,
Martina Stary,
Georg Weitzer
Max F. Perutz Laboratories, Department of Medical, Biochemistry, Medical University of Vienna, Dr. Bohrgasse 9, A1030 Vienna, Austria

Abstract

Desmin contributes to structural integrity and function of the myocardium but its function seems to be redundant in early cardiomyogenesis in the desmin null mouse model. To test the hypothesis that desmin also plays a supportive role in cardiomyogenic commitment and early differentiation of cardiomyo-cytes we investigated cardiomyogenesis in embryoid bodies expressing different *desmin* alleles. Constitutive expression of *desmin* and increased synthesis during mesoderm formation led to the up-regulation of *brachy-ury* and *nkx*2.5 genes, accelerated early cardiomyo-genesis and resulted in the development of large, proliferating, highly interconnected, and synchronously beating cardiomyocyte clusters, whereas desmin null cardiomyocytes featured an opposite phenotype. In contrast, constitutive expression of amino-terminally truncated *desmin*$^{\Delta 1-48}$ interfered with the beginning of cardiomyogenesis, caused down-regulation of mesodermal and myocardial transcription factors, and hampered myofibrillogenesis and survival of cardiomyocytes. These results provide first evidence that a type III intermediate filament protein takes part in regulating the differentiation of mesoderm to cardiomyocytes at the very beginning of cardiomyogenesis.

Keywords

cardiomyogenesis; desmin; nkx2.5; brachyury; embryoid body

Introduction

Structural integrity and rhythmic excitation of cardiomyocytes is based on a complex cellular system composed of the contractile apparatus, cytoskeleton, membrane skeleton, sarcoplasmic reticulum, and extracellular matrix (Clark et al., 2002). A prominent member of the first three of these compartments is desmin, the subunit of the muscle specific type

Correspondence to: Georg Weitzer.
Max F. Perutz Laboratories, Department of Medical, Biochemistry, Medical University of Vienna, Dr. Bohrgasse 9, A1030 Vienna, Austria, Tel: +43 1 4277 616 50, Fax: +43 1 4277 9616, georg.weitzer@meduniwien.ac.at.

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III intermediate filaments (Capetanaki, 2000; Costa et al., 2004; Paulin and Li, 2004). Expression of desmin becomes visible in murine precardiac mesoderm at day 7.25 (Kuisk et al., 1996), emanating from *brachyury* and *goosecoid* expressing mesodermal precursors (Olson, 2004). Additionally, desmin mRNA was detected in embryoid bodies (EBs) at day 5 (Weitzer et al., 1995) suggesting a role in early embryonic development before cardiomyogenesis takes place. Later in development *desmin* is also expressed in skeletal and smooth muscle cells and influences myogenesis at the transcriptional level by modulating the expression of the skeletal muscle specific bHLH transcription factor genes *myogenin, myoD*, and *myf*5 (Li et al., 1994; Weitzer et al., 1995), however, so far appeared to be dispensable for cardiomyogenesis *in vitro* and *in vivo* (Weitzer et al., 1995; Li et al., 1996; Milner et al., 1996).

Mice lacking desmin develop lesions in cardiac muscle (Li et al., 1996; Milner et al., 1996) which are accompanied by the disorganization of myofibrils, intercalated discs, and mitochondria (Thornell et al., 1997; Balogh et al., 2002; Capetanaki, 2002). The cardiac phenotype can be partially overcome by the expression of *bcl*-2, rescuing the mitochondrial phenotype (Weisleder et al., 2004b) and by expression of *desmin* under the control of the *mhc*α promoter (Weisleder et al., 2004a). In skeletal muscle, lack of desmin results in lower active force generation, improves fatigue resistance, decreases isometric stress production and reduces vulnerability to mechanical injury (Li et al., 1997; Sam et al., 2000; Balogh et al., 2003; Shah et al., 2004). An amino-terminally deleted desmin rescued myogenesis in skeletal muscle but compromised cardiomyogenesis in EBs (Höllrigl et al., 2002). Finally, in smooth muscle cells desmin has a role in cellular transmission of both active and passive force (Sjuve et al., 1998; Shardonofsky et al., 2006).

Early expression of *desmin* in precardiac mesoderm (Kuisk et al., 1996) and its influence on transcription of early myogenic transcription factor genes in skeletal muscle (Li et al., 1994; Weitzer et al., 1995) suggests a role for desmin in very early developmental processes such as cardiomyogenic commitment and differentiation. While substantial progress has been made in clarifying desmin's contribution to function, stability and longevity of the working myocardium, evidence supporting a role in early cardiomyogenesis is lacking. Cardiomyogenesis at its very beginning is supposed to start in the splanchnic mesoderm beneath the developing head fold under the transcriptional control of the T-box, tinman/nkx, and gata families of transcription factors (Hiroi et al., 2001; Masino et al., 2004).

Here, we test the hypothesis that desmin affects very early cardiomyogenesis. To this end we investigated *in vitro* cardiomyogenesis in EBs, generated from embryonic stem cells (ESCs) constitutively expressing an additional wild-type *desmin* allele, and from ESCs constitutively expressing two mutant *desmin* alleles in a null background, giving rise to amino-terminally deleted desmin$^{\Delta 1-48}$. We found that constitutive expression of desmin indeed promoted early cardiomyogenesis and up-regulates the expression of the *brachyury* and *nkx2.5* genes, whereas absence of desmin delayed cardiomyogenesis, and constitutive expression of *desmin*$^{\Delta 1-48}$ negatively affected early cardiomyogenesis and transcription factor gene expression.

Materials and methods

Generation of ESC lines

Des$^{-/-}$ and *des*$^{\Delta 1-48/\Delta 1-48}$ ESCs were generated as described (Weitzer et al., 1995; Höllrigl et al., 2002). The desmin expression vector was constructed by inserting the murine desmin cDNA (Li and Capetanaki, 1993) into pBK-RSV (Stratagene, La Jolla, CA), and verified by sequencing. Transfection of AB2.2 ESCs with pBK-RSV-Desmin was performed by electroporation and selection for random integration with 180 μg/ml G418.

In vitro differentiation of ESCs and analysis of cardiomyogenesis in EBs

Each 800 ESCs were aggregated in 20 μl medium drops for 4 days, 80 ± 10 EBs of equal size were plated onto gelatinized 10 cm tissue culture dishes and cardiomyogenesis was monitored essentially as described (Weitzer et al., 1995; Bader et al., 2000). Development of cardiomyocytes was compared by calculating the maximal daily increase in the number of beating cardiomyocytes and the maximal percentage of EBs with beating cardiomyocytes at the day of maximum beating activity ± 2 days. Clusters of beating cardiomyocytes in EBs were counted and classified as small, when composed of less than 10, or as large, when composed of more than 10 cardiomyocytes. Number of clusters directly reflects commitment and size of clusters reflects proliferation of cardiomyocytes (Lauss et al., 2005).

Immunofluorescence microscopy

Immunostaining of EBs was performed with desmin antibodies D8281 (Sigma-Aldrich, Vienna, Austria), Y20 (Santa Cruz Biotechnology, Heidelburg, Germany), and with cardiac troponin T (cTnT) antibodies (Neomarkers, Vienna, Austria), followed by FITC- and TRITC-conjugated secondary antibodies (Sigma F4018, T-5268). The annexin V binding assay was performed according to supplier's protocol (1858777, Roche, Vienna, Austria). For terminal deoxynucleotidyl transferase-mediated dNTP-fluorescein nick end labeling (TUNEL) assays EBs were fixed in 4% para-formaldehyde solution, permeabilized and stained for cTnT, in this case using a Texas red-conjugated secondary antibody (Jackson Immuno research, Suffolk, UK). TUNEL assay was performed according to supplier's protocol (1684809, Roche) followed by DAPI staining. Photomicrographs were taken from areas where typically cardiomyogenesis takes place in EBs (Weitzer, 2006) on a Leica TCS SP confocal microscope (Wetzlar, Germany) with × 40 and × 100 objectives and on a Zeiss Axiovert LSM 510 microscope (Vienna, Austria) with an × 63 objective. Images were prepared by Adobe Photoshop 7.0.

Fluorescence activated cell sorting analysis

Eighty EBs of each genotype were allowed to develop until day 5, then cells were separated by digestion with trypsin for 10 min at 37°C. 500,000 cells were resuspended in PBS, dead cells were stained with propidium iodide (10 μg/ml) and sorted in a BD Becton&Dickinson LSR1 fluorescence activated cell sorter (Schwechat,Austria).

Western blot analysis

Western blot analysis was performed with intermediate filament preparations from EBs (Weitzer and Wiche, 1987) using anti-desmin (Y20, Santa Cruz Biotechnology), anti-vimentin (ICN, Eschwepe, Germany), and anti-connexin 43 (Sigma-Aldrich) antibodies and secondary alkaline phosphatase conjugated antibodies (Promega, Mannheim, Germany).

Semi-quantitative RT-PCR analysis

mRNA was prepared from ESCs and EBs with the RNeasy Mini Kit (Qiagen, Vienna, Austria) and reverse transcribed with Superscript II polymerase (Invitrogen, Merelbeke, Belgium) in at least three independent experiments. Polymerase chain reaction (PCR) was performed with primer pairs as indicated in Figures 1 and 6. For semi-quantitative PCR samples were diluted 1:0 1:10, 1:100, and 1:1,000. Primer sequences and parameters of amplification are available on request. Number of cycles were carefully determined by several preparatory experiments and chosen so that none of the obtained signals were saturated. RSV promoter driven expression of desmin was determined by using a forward primer situated in the RSV promoter, endogenous expression was detected with a reverse primer situated in the 3′ UTR not contained in the des^{ect} allele. Statistical analysis of the *brachyury* and *nkx2.5* expression was performed by measuring the luminosity of ethidiumbromide stained RT-PCR products by Adobe Photoshop 7.0 tools of six independent experiments.

Statistical analysis

All data are presented as the arithmetic mean ± standard deviation $\sigma_{x(n-1)}$. Statistical significance was evaluated using one sample and paired samples Student's *t*-test, respectively and values of $p < 0.05$ were considered to indicate statistical significance.

Results

Constitutive expression and synthesis of desmin and desmin $^{\Delta 1-48}$ is maintained throughout cardiomyogenesis in EBs

Transfection of ESCs with a desmin expression cassette under the control of the constitutive active RSV promoter resulted in desmin protein synthesis detectable in intermediate filament preparations by Western blot analysis (Fig. 1A, lane 1). Constitutive expression and protein synthesis of desmin $^{\Delta 1-48}$ and absence of desmin in $des^{-/-}$ ESCs were previously described (Weitzer et al., 1995; Höllrigl et al., 2002). Constitutive expression of *desmin* was maintained in EBs throughout the time of cardiomyogenesis (Fig. 1B). Constitutive expression of an additional *desmin* allele resulted in an increased desmin protein level in $des^{+/+}$ des^{ect} EBs, whereas in *des* $^{\Delta 1-48/\Delta 1-48}$ EBs the desmin $^{\Delta 1-48}$ protein level was similar to that in $des^{+/+}$ EBs (Fig. 1C). Neither vimentin nor connexin 43 protein levels were affected by the expression of different *desmin* alleles.

Desmin accelerates differentiation of cardiomyocytes whereas mutant desmin$^{\Delta 1-48}$ negatively affects cardiomyogenesis in Ebs

Constitutive synthesis of desmin in differentiating ESCs induced a 24 ± 4 hr earlier onset of cardiomyogenesis in *des*$^{+/+}$*des*^{ect} EBs (Fig. 2A, arrow), normally starting at day 7 ± 6 hr in wild-type *des*$^{+/+}$ EBs, caused an accelerated development of cardiomyocytes (Fig. 2B), and resulted in large areas of highly proliferating cardiomyocytes in 100% of EBs (Fig. 2C). Absence of any effect of conditioned cell culture supernatant from *des*$^{+/+}$*des*^{ect} EBs on cardiomyogenesis in wild-type EBs (data not shown) suggests that desmin does not influence cardiomyogenesis by a paracrine loop via other cell types but acts in a cell autonomous manner. In the absence of desmin, development of cardiomyocytes was delayed by 1 day (Fig. 2A) but rate of differentiation was slightly affected (Fig. 2B). Maximum number of EBs with beating cardiomyocytes was only insignificantly decreased (Fig. 2C), however, the size of cardiomyocyte clusters was much smaller. In order to test the effect of a truncated and potentially dominant-negative mutant molecule in the onset of cardiomyogenesis, differentiation of cardiomyocytes was investigated in ESCs expressing a mutant desmin protein lacking its amino-terminal domain in a null background. Constitutive synthesis of desmin$^{\Delta 1-48}$ caused a 2 days delay of cardiomyogenesis (Fig. 2A), a drastically reduced differentiation rate (Fig. 2B), and very low number of EBs with small clusters of cardiomyocytes (Fig. 2C). Notably, this phenotype was much worse than that observed in the absence of desmin in *des*$^{-/-}$ EBs and suggests a functional involvement of desmin in commitment and early differentiation of cardiomyocytes.

Desmin facilitates proliferation and maturation of cardiomyocytes

To investigate the influence of desmin and desmin$^{\Delta 1-48}$ on the morphology of cardiomyocytes, EBs were stained with desmin antibodies and cardiomyocytes were identified with antibodies to cTnT. Compared with wild-type, *des*$^{+/+}$ EBs, the size of cardiomyocyte clusters was significantly decreased in *des*$^{-/-}$ EBs but enormously increased in *des*$^{+/+}$*des*^{ect} EBs (Fig. 3A). Frequently the total central area of *des*$^{+/+}$*des*^{ect} EBs was covered with an interwoven synchronously beating network of cardiomyocytes, suggesting that overexpression of *desmin* also increased proliferation of cardiomyocytes. In *des*$^{\Delta 1-48/\Delta 1-48}$ EBs cluster size was reduced to that observed in *des*$^{-/-}$ EBs again demonstrating a dominant-negative effect of desmin$^{\Delta 1-48}$.

At high magnification it became evident that absence of desmin did not significantly alter the number of myofibrils in differentiating cardiomyocytes but expression of desmin$^{\Delta 1-48}$ drastically inhibited formation of myofibrils with regular sarcomeres and caused a diffuse distribution of cTnT (Fig. 3B). Most *des*$^{\Delta 1-48/\Delta 1-48}$ cardiomyocytes had an aberrant structure and seemed to be less connected to each other, again demonstrating that truncation of the amino terminus of desmin is worse than the lack of desmin in cardiomyocytes. By contrast, overexpression of desmin resulted in enlarged and very dense array of cardiomyocytes with numerous myofibrils with regularly aligned sarcomeres.

To assess the influence of desmin on early proliferation of still differentiating cardiomyocytes, we measured the number and size of cardiomyocyte clusters shortly before cardiomyogenesis reached maximum levels in EBs. Constitutive synthesis of desmin resulted in increased numbers of cardiomyocyte clusters within all EBs (Fig. 4A), and in an extreme preponderance of large over small clusters of cardiomyocytes (Fig. 4B).

Synchronous beating of all cardiomyocytes with significantly increased contraction rates in *des*$^{+/+}$*des*^{ect} EBs suggest a physiological interaction of these cardio-myocytes (Fig. 4C). In the absence of desmin cluster size shifted to small not synchronously contracting aggregates (Fig. 4B), with unchanged contraction rates (Fig. 4C). In the presence of desmin$^{\Delta 1-48}$ number and size of cardiomyocyte clusters was drastically reduced (Figs. 4A,4B), and contraction rates (Fig. 4C) were dramatically lowered. In *des*$^{\Delta 1-48/\Delta 1-48}$ cardiomyocytes contraction became arrhythmic with increasing age (data not shown).

Low numbers and small size of cardiomyocyte clusters might be also due to increased apoptosis of cardiomyocytes expressing desmin$^{\Delta 1-48}$. FACS analysis of EBs stained for dying or dead cells with propidium iodide at day 5 when primitive mesoderm forms demonstrated a weak but significant increase in the number of dying cells in *des*$^{\Delta 1-48/\Delta 1-48}$ EBs (Table 1). This finding was in line with very similar results obtained with EBs expressing a dominant-negative allele of desmin under the control of the desmin promoter (Höllrigl et al., 2007) Thus, it is likely that desmin$^{\Delta 1-48}$ affects apoptosis of some mesodermal precursors for those un-fortunately still no specific markers exist. To demonstrate a specific influence on cardiomyocytes, we performed TUNEL and annexin V binding assays in EBs on day 8, the first day when cardiomyocytes appear in *des*$^{\Delta 1-48/\Delta 1-48}$ EBs. Cardiomyocytes of all genotypes had nearly no TUNEL-positive nuclei at day 8 (Fig. 5A). Only two of 1083 cTnT-positive *des*$^{+/+}$ cardiomyocyte had TUNEL assay positive nuclei or annexin V bound to its plasma membrane (Fig. 5A, inset). No cTnT-positive *des*$^{\Delta 1-48/\Delta 1-48}$ cardiomyocytes were TUNEL positive ($N = 109$). Annexin V binding assay corroborated the very low susceptibility of cTnT-positive cardiomyocytes to apoptosis on day 8 (data not shown). At day 12 again no signs of apoptosis in cardiomyocytes were observed (data not shown). At day 16, however, when *des*$^{\Delta 1-48/\Delta 1-48}$ cardiomyocytes prematurely seize to contract (see Fig. 2A) a significantly increased number of TUNEL-positive nuclei (25%, $N = 60$) was observed along with a clearly degenerated morphology (Fig. 5B). Thus, desmin$^{\Delta 1-48}$ affects most likely pre-myocardial mesoderm and committed and differentiated cardiomyocytes. However, from these data it cannot be excluded that desmin$^{\Delta 1-48}$ also affects proliferation of committed cardiomyocytes. An increased number of TUNEL-positive nuclei were also found in the neighborhood of cTnT-positive cardiomyocytes in *des*$^{-/-}$ EBs, however, apoptosis in *des*$^{+/+}$ and *des*$^{+/+}$*des*^{ect} cardiomyocytes remained low in the range of 5−10% ($N = 218$ each). Together these data strongly suggest that desmin actively facilitates commitment, proliferation and differentiation of cardiomyocytes.

Desmin transiently promotes the expression of mesodermal transcription factor brachyury and early myocardial transcription factor nkx2.5

To provide molecular evidence for desmin's positive influence on the early differentiation of cardiomyocytes we investigated the expression of genes specific for mesoderm and cardiomyocytes by semi-quantitative RT-PCR from days 0 to 8 of *in vitro* development (Fig. 6A). Changes in the expression pattern of cardiac muscle markers are specific for cardiomyogenesis because skeletal and smooth muscle cells start to differentiate much later in EB development (Weitzer et al., 1995). In wild-type EBs *desmin* transcripts could be detected at the beginning of mesoderm development at day 4 and absence of

desmin in EBs had a weak negative effect on the expression of *brachyury*, and caused a significant delay in *mlc*1*v* expression (Fig. 6, third lanes), indicative of delayed or hampered cardiomyocyte maturation (Trahair et al., 1993), which was also evident from the small size of cardiomyocyte clusters (see Fig. 3A). Constitutive desmin$^{\Delta 1-48}$ synthesis caused the partial downregulation of mesodermal transcription factor genes *brachyury* and *goosecoid*, and the complete lack of early myocardial transcription factor genes *nkx2.5* and *mef2C* expression between days 4 and 6 (Fig. 6A, second lanes). Expression of the cardiomyocyte specific gene *mlc*1*v* was delayed by 2 days and *mhc*α, also expressed in other cell types at later stages of development was not significantly affected. Expression of vimentin, a candidate protein to compensate desmin was not significantly altered in EBs of any genotype. As suggested by the delayed and reduced development of cardiomyocytes in EBs, desmin$^{\Delta 1-48}$ indeed affects mesodermal precursors of cardiomyocytes and differentiating cardiomyocytes.

Constitutive overexpression and synthesizes of desmin promoted differentiation of ESCs as evident from the earlier down-regulation of the stemness transcription factor gene *oct*3/4, and significantly promoted the expression of primitive mesoderm markers *brachyury* and *goosecoid*. Concomitantly within the limits of resolution, *nkx2.5* were transiently up-regulated between days 4 and 6 followed by the transient up-regulation of *mlc*1*v* and *mhc*α on day 6 (Fig. 6A, fourth lanes). Statistical analysis of *brachyury* and *nkx2.5* expression between day 4 and 6 in six independent experiments demonstrates a significant influence of desmin on *brachyury* expression in precardiac mesoderm and on *nkx2.5* expression in early differentiating cardiomyocytes (Figs. 6B, 6C), suggesting that desmin influences cardiomyogenesis already in the primitive mesoderm before cardioblasts differentiate to functional cardiomyocytes.

Discussion

This study demonstrates an involvement of desmin in mechanisms regulating the very beginning of cardiomyogenesis and the expression of transcription factor genes important for mesodermal and early myocardial differentiation.

RSV promoter driven constitutive expression of desmin in ESCs before mesodermal cells become committed to cardiomyogenic lineages induced premature onset of cardiomyogenesis in EBs and resulted in 100% EBs with large and interwoven areas of cardiomyocytes. Mesodermal precursors of cardioblasts differentiated much faster in the presence of desmin. Cardiomyocytes were synchronously beating at twice the rate observed in wild-type EBs and had a perfect differentiated phenotype. Additionally, cardiomyocyte cluster size was significantly increased, which is indicative of increased proliferation of differentiating cardiomyocytes (Lauss et al., 2005), and suggesting that desmin not only fosters commitment but also proliferation of differentiating cardiomyocytes. Absence of any effect of conditioned cell culture supernatant from *des*$^{+/+}$*des*^{ect} EBs on cardiomyogenesis in wild-type EBs suggests that desmin influences cardiomyogenesis in a cell autonomous manner. In the absence of desmin exactly the opposite phenotype was observed. Cardiomyogenesis was delayed by 1 day and cardiomyocyte clusters were

composed of much less cells which did not beat synchronously and thus seem to be less well connected to each other.

Overexpression of desmin under the control of cardiac specific promoters activated only in committed cardiomyocytes (Wang et al., 2001; Haubold et al., 2003; Weisleder et al., 2004a) had no discernable effects on cardiomyocyte commitment and differentiation. This suggests that indeed it is the expression in mesodermal precursors that triggers increased cardiomyogenesis and subsequent formation of large clusters of cardiomyocytes, and not simply the fact that desmin is expressed from three instead of two alleles in cardiomyocytes.

The influence of desmin on the commitment of mesoderm to cardiomyogenic lineages was further supported by increased expression of mesodermal transcription factor genes *brachyury* and *goosecoid* and the faster down-regulation of the stemness gene *oct*3/4 in the presence of desmin. Concomitantly expression of the gene of the early myocardial transcription factor Nkx2.5 was up-regulated which led to the transient up-regulation of its downstream target gene *mef2C* (Jamali et al., 2001) and the *mlc*1 and mhcα genes. In line with these results, the *desmin* gene was expressed in EBs at day 4, at a time when first primitive mesodermal cells emerge from the inner primitive ectoderm of EBs (Kubo et al., 2004), and vice versa, absence of desmin caused a temporally reduced expression of *brachyury* and delayed the expression of *mlc*1*v*. Notably, at this developmental stage both *in vitro* and *in vivo* cardiomyocytes are the only differentiating muscle cells and are also the only cells expressing markers such as *nkx2.5* and *mef2C*. This excludes a contribution to the observed expression patterns by other cell types. T-box transcription factors together with Nkx2.5 are known to synergistically promote cardiogenesis (Hiroi et al., 2001), and desmin has been shown to directly bind to DNA via its amino-terminus (Li et al., 2003; Tolstonog et al., 2005), thus it seems likely that desmin promotes cardiomyogenesis via the up-regulation of brachyury and nkx2.5.

In vivo desmin expression was detected in the premyocardial splanchnic mesoderm (Kuisk et al., 1996) lending physiological relevance to desmin's influence on the expression of mesodermal and early myocardial transcription factor genes and its supporting role during early cardiomyogenesis in EBs *in vitro*. Having said that, these data also clearly demonstrate that desmin *perse* does not drive ESCs into the cardiomyogenic lineage. Pre-gastrulation-like development of extra-embryonic and primitive ectodermal lineages in EBs (Weitzer, 2006) seems to be indispensable for *in vitro* cardiomyogenesis in providing a still unknown set of specification factors secreted by other cell types.

To further support the hypothesis that desmin influences commitment and very early differentiation of cardioblast, we studied the effects of a mutant desmin protein negatively affecting cardiomyogenesis (Höllrigl et al., 2002). This allows determining whether desmin, not being detectable at this developmental stage *in vivo*, has to be functional at the time when mesodermal precursors are commitment to the cardiomyogenic lineages.

Indeed, homozygous expression of $desmin^{\Delta 1-48}$ in the absence of wild-type desmin caused a significant delay and attenuation of cardiomyogenesis in EBs. $Desmin^{\Delta 1-48}$ likely causes death of some mesodermal cells in day 5 EBs, however, may as well negatively influence

proliferation of these cells. In addition, desmin$^{\Delta 1-48}$ partially inhibited expression of *brachyury* and *goosecoid* in mesodermal cells at day 4, and completely inhibited expression of *nkx2.5, mef2C* and *mlc1v* in differentiating cardiomyocytes at day 6. This demonstrates that mutant desmin influences developmental processes involved in the differentiation of cardiomyocytes significantly before genes of typical structural proteins like MHCα are expressed. Most importantly, desmin$^{\Delta 1-48}$ caused a phenotype which was much worse than that observed in *des*$^{-/-}$ EBs suggesting that this mutant protein actively interferes with the onset of cardiomyogenesis. The amino terminus of desmin, vimentin and glial fibrillary acidic protein has been demonstrated to mediate the interaction of cytoplasmic IF proteins with single-strand and double-strand DNA (dsDNA) (Shoeman et al., 2001; Shoeman et al., 2002; Tolstonog et al., 2005), thus desmin$^{\Delta 1-48}$ may well lack this function which might be important for differentiation of cardiomyocytes. Integration of too much mutant protein into type III filaments might alter their propensity to disassemble before cytokinesis in cardiomyocytes. However, toxicity of the mutant protein *per se* has been previously excluded by the fact that cardiomyogenesis and myogenesis was not affected in *des*$^{+/\Delta 1-48}$ EBs (Höllrigl et al., 2002). Deregulation of myocardial differentiation and morphogenesis in *des*$^{\Delta 1-48/\Delta 1-48}$ EBs may be either a consequence of a pathological function of the desmin protein lacking 48 amino-terminal amino acid residues, or due to the lack of an important function situated in the amino-terminal domain of desmin which is known to interact with several cytoskeletal proteins, such as ankyrin, plectin, and desmoplakin (Costa et al., 2004) and with dsDNA (Tolstonog et al., 2005).

During further development of *des*$^{\Delta 1-48/\Delta 1-48}$ EBs, however, the number of cardiomyocyte clusters and the number of cells within the clusters was drastically reduced, the later suggesting a negative effect of desmin$^{\Delta 1-48}$ on proliferation or survival of differentiating cardiomyocytes. Cardiomyocytes had an aberrant morphology featuring reduced numbers of regular myofibrils. Sarcomere length in those myofibrils still forming was significantly reduced from an average of 1.98 ± 0.19 μm (N = 552) in *des*$^{+/+}$ *des*^{ect}, *des*$^{+/+}$, and *des*$^{-/-}$ cardiomyocytes to 1.46 ± 0.25 mm (N= 225, p = 0.0001) in *des*$^{\Delta 1-48/\Delta 1-48}$ cardiomyocytes. Although desmin$^{\Delta 1-48}$ rescued fusion of *des*$^{-/-}$ myoblasts (Höllrigl et al., 2002), it actively interfered with myofibrillogenesis in cardiomyocytes and caused reduced and arrhythmic contraction. Additionally, desmin formed aggregates, which co-stained for cTnT, suggesting that mutant desmin causes myofibril proteins to aggregate. Aggregation of myofibril proteins has been previously reported to cause apoptosis. Fragments of desmin induce apoptosis in cardiomyocytes (Chen et al., 2003) and desmin aggregation also impairs proteolytic function of cardiomyocytes (Liu et al., 2006). In accordance with these results we observed an increased rate of apoptosis in *des*$^{\Delta 1-48/\Delta 1-48}$ EBs at day 16 when *in vitro* cardiomyogenesis comes to a halt and cardiomyocytes slowly stop to contract in wild-type EBs. Only a small number of cTnT-positive cardiomyocytes had TUNEL assay-positive nuclei but a significant number of nuclei in neighboring cells were TUNEL assay positive or showed signs of micronuclei formation and nuclear chromatin aggregation. Because of the defined location of these cells within EBs (Weitzer, 2006) it is very likely that these cells had been cardiomyocytes which had already lost their cTnT protein in the course of apoptosis. Finally, in agreement with findings in desmin null mice (Weisleder et al., 2004b) apoptosis was also increased in matured *des*$^{-/-}$ cardiomyocytes, supporting the current working model

for desmin as an essential protein for structural integrity and function of cardiomyocytes (Capetanaki, 2002; Paulin and Li, 2004) and suggesting that the integrity of the amino-terminal domain which mediates desmin's interaction with DNA is key to the proper function of desmin in terminally differentiated cardiomyocytes.

In conclusion, we have found that desmin influences early cardiomyogenesis in EBs at the molecular and cellular level. Desmin modulates the expression of mesodermal and early myocardial genes and promotes proliferation of differentiating cardiomyocytes in addition to its well-documented role in the maintenance of the muscle cell phenotype. These data provide evidence to the hypothesis that desmin has a supportive role during commitment and early differentiation of the cardiomyocytes.

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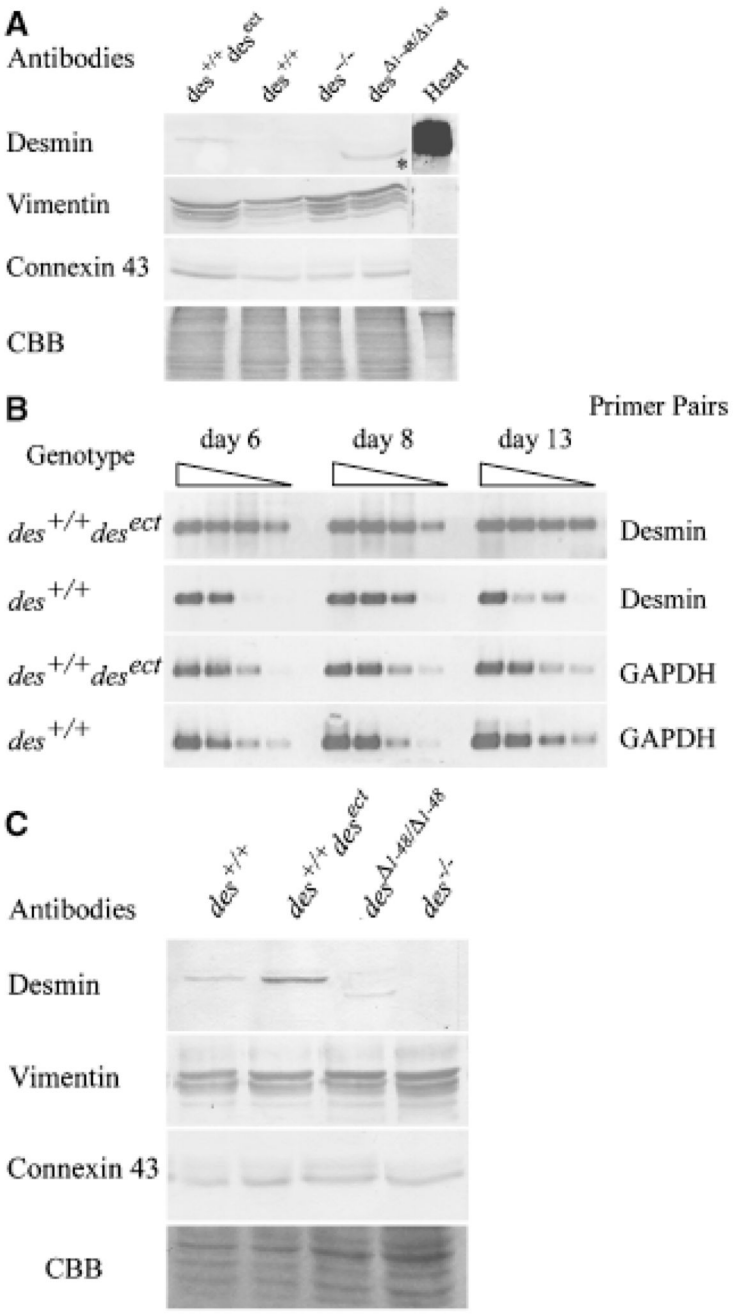

Fig. 1. Expression and synthesis of desmin and desmin$^{\Delta 1-48}$ in embryonic stem cells and embryoid bodies (EBs).

(**A**) Western blot analysis of intermediate filament preparations from embryonic stem cells with an additional ectopic allele constitutively expressing *desmin* (*des*$^{+/+}$ *des*^{ect}), two wild-type *desmin* alleles (*des*$^{+/+}$), two null alleles (*des*$^{-/-}$), and two constitutively expressed mutant alleles lacking codons 1−48 (*des*$^{\Delta 1-48/\Delta 1-48}$), with antibodies against desmin, vimentin, and connexin 43. Heart: positive control. Connexin 43 immuno-blot and CBB, Coomassie brilliant blue stained gel, loading controls. *Note, smaller size of the

constitutively synthesized desmin lacking amino acids 1−48. (**B**) Semi-quantitative RT-PCR of *des*$^{+/+}$ and *des*$^{+/+}$ *des*^{ect} EBs with *desmin* and *GAPDH* primer pairs at different stages of EB development. Rows 1−4 represent steps of tenfold dilution of the cDNA for each day. (**C**) Expression of wild type, ectopic and mutant desmin proteins in EBs. Western blot analysis of intermediate filament preparations from EBs at day 16. Antibodies and genotypes as indicated.

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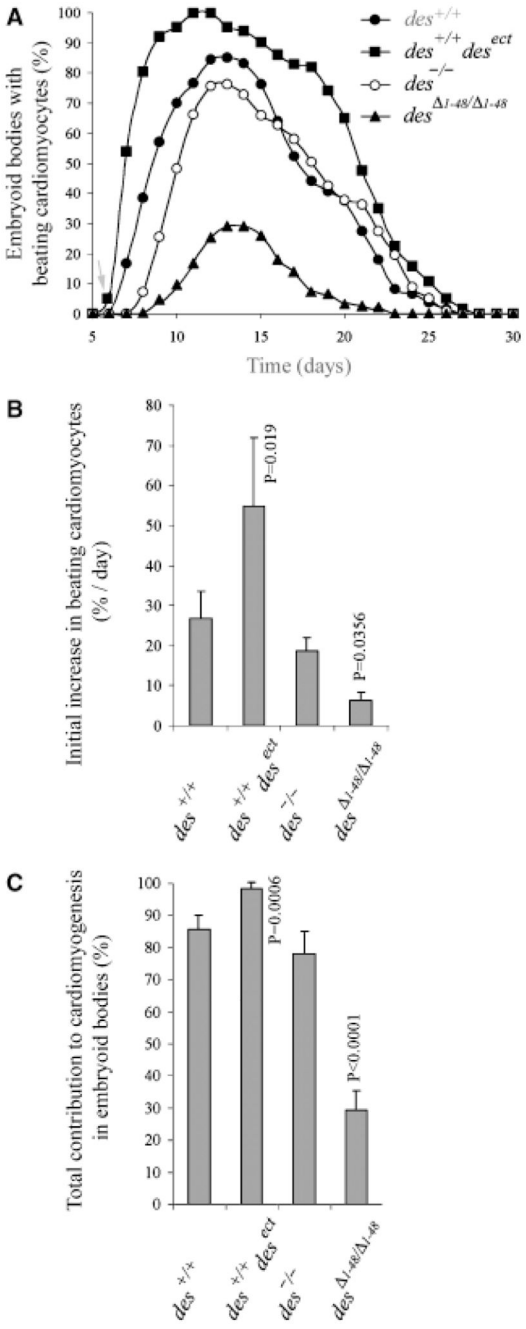

Fig. 2. Premature synthesis of desmin causes early onset of cardiomyogenesis in embryoid bodies (EBs), but lack of its amino-terminal domain severely hampers cardiomyogenesis.

(**A**) Development of beating cardiomyocytes in EBs generated from embryonic stem cells of genotypes as indicated. Note, that only $des^{+/+}des^{ect}$ cardiomyocytes started to beat on day 6 (arrow) and onset of cardiomyogenesis in $des^{\Delta1-48/\Delta1-48}$ EBs was delayed by 2 days. (**B**) Acceleration of development to beating cardiomyocytes by desmin. Starting 1 day after the first beating cardiomyocytes were observed, increase of beating cardiomyocytes per day was measured for 3 consecutive days. (**C**) Influence of desmin on the extent of cardiomyogenesis

in EBs. Means were calculated from the day of maximum beating activity ± 2 days. (A−C) Data from experiments with two *des*$^{+/+}$ *des*^{ect} clones were indistinguishable and thus combined. Data are means of six independent experiments. Number of EBs analyzed in each case: $N = 386$. Error bars, standard deviation $\sigma_{x(n-1)}$. Denoted significant p-values relate to control (*des*$^{+/+}$).

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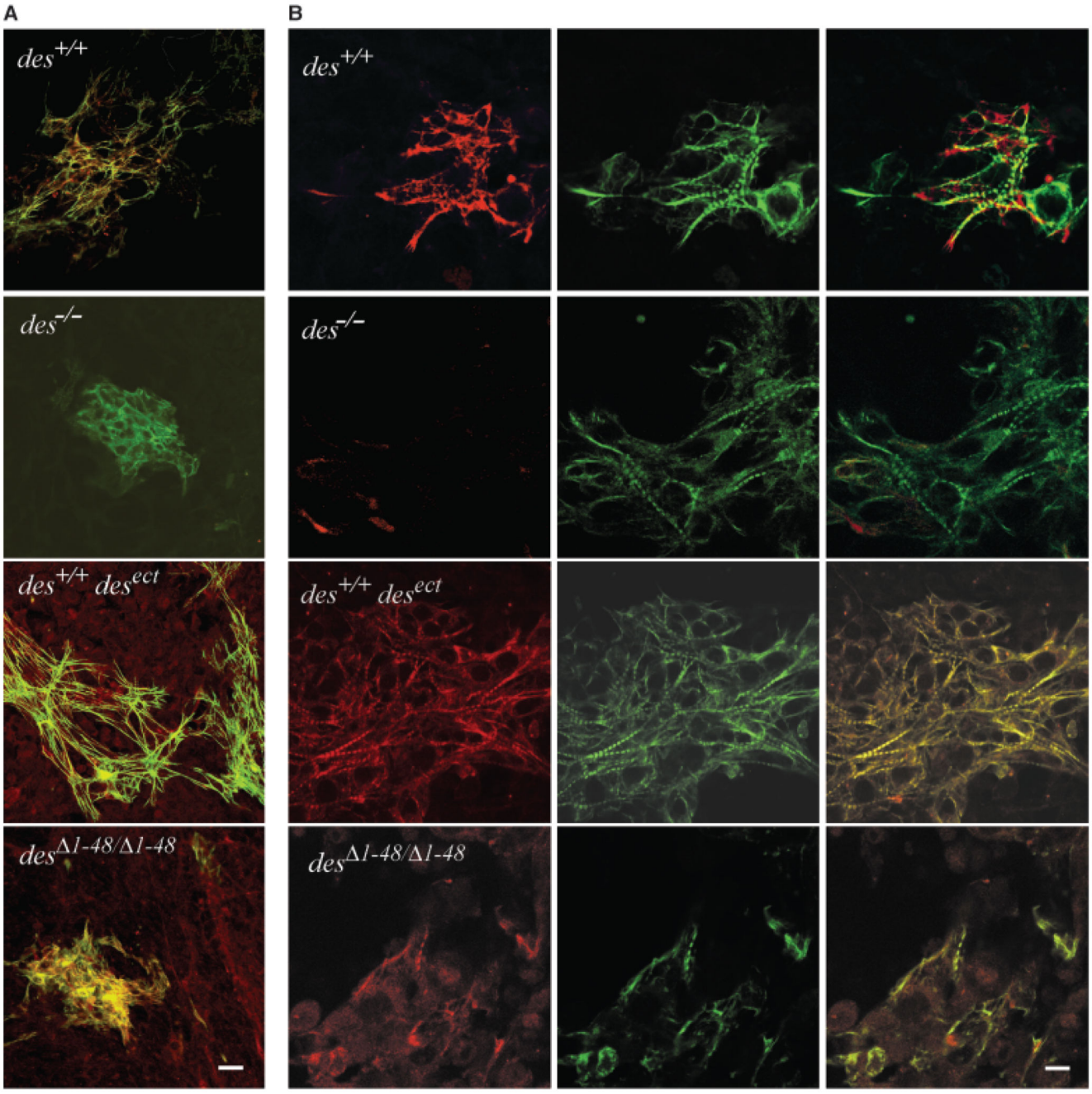

Fig. 3. Desmin increases size and interconnections of cardiomyocyte clusters but desmin$^{\Delta 1-48}$ causes disorganization of contractile apparatus.

(**A**) Merged confocal double immunofluorescence micrographs of typical cardiomyocyte clusters in embryoid bodies (EBs) with desmin alleles as indicated. EBs were double stained with a polyclonal antibody to desmin and a secondary TRITC-conjugated antibody (red) and a monoclonal antibody to cTnT and a secondary FITC-conjugated antibody (green) at day 16 after embryonic stem cells aggregation. Note, ectopic synthesis of desmin in all cells of *des*$^{+/+}$ *des*^{ect} and *des*$^{\Delta 1-48/\Delta 1-48}$ EBs. Scale bar, 25 μm. (**B**) High-resolution

confocal double immunofluorescence micrographs of cardiomyocytes stained as in (A). Right column, merged images. Scale bar, 10 μm.

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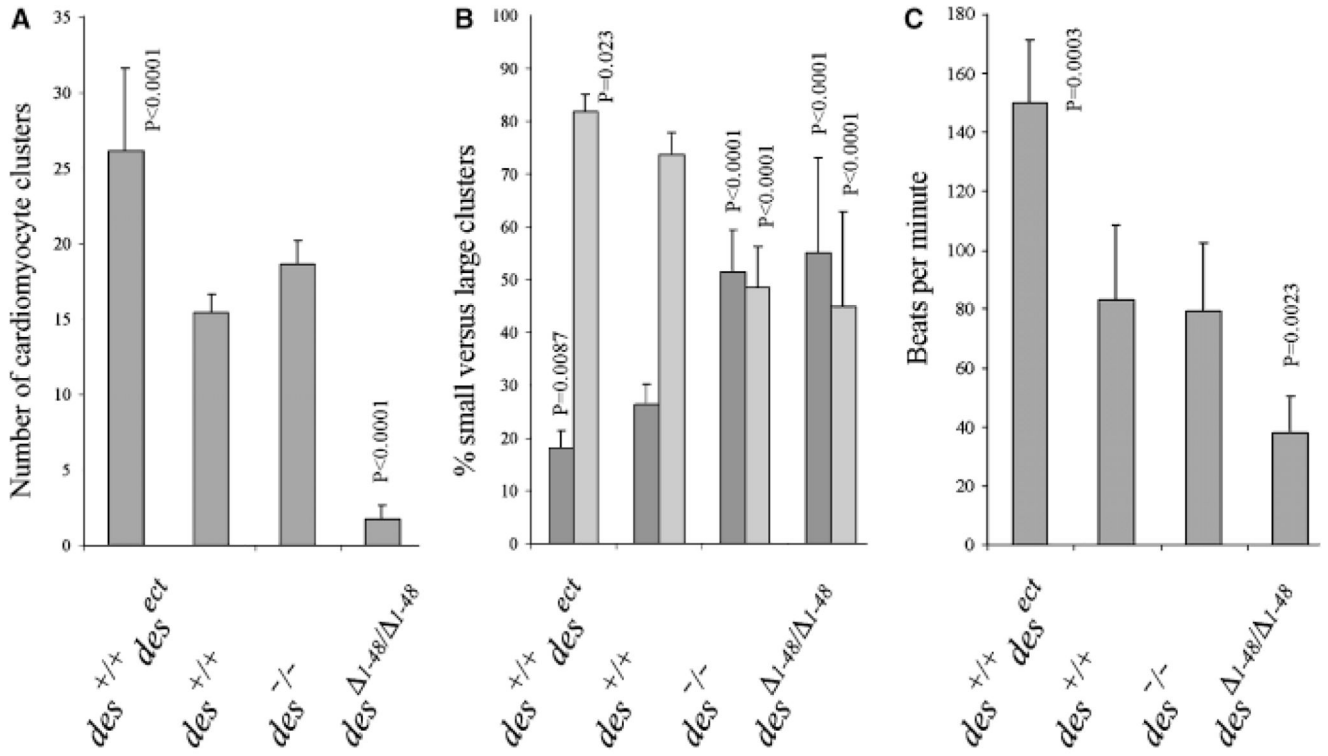

Fig. 4. Desmin promotes commitment and proliferation of cardiomyocytes in embryoid bodies (EBs).

(**A**) Number of beating cardiomyocyte clusters per EB indicating commitment of cardiomyocytes. Number of EBs analyzed in each case, $N = 311$; except for $des^{+/+}$ and $des^{+/+}des^{ect}$, $N = 916$. (**B**) Size distribution of cardiomyocyte clusters which is directly proportional to the proliferation of differentiating cardiomyocytes, presented as the relative proportion of small clusters with <10 (dark gray bars) versus large clusters with up to several hundred cardiomyocytes (light gray bars). Number of EBs analyzed in each case: $N = 386$. (**C**) Rhythmic contraction of cardiomyocytes was measured on day 16 ± 4. Number of cardiomyocyte clusters analyzed: $des^{+/+}$, $N = 85$; $des^{+/+}des^{ect}$, $N = 109$; all others $N = 52$. Data are means of six independent experiments. Error bars, standard deviation $\sigma_{x(n-1)}$. Denoted significant p-values relate to control ($des^{+/+}$).

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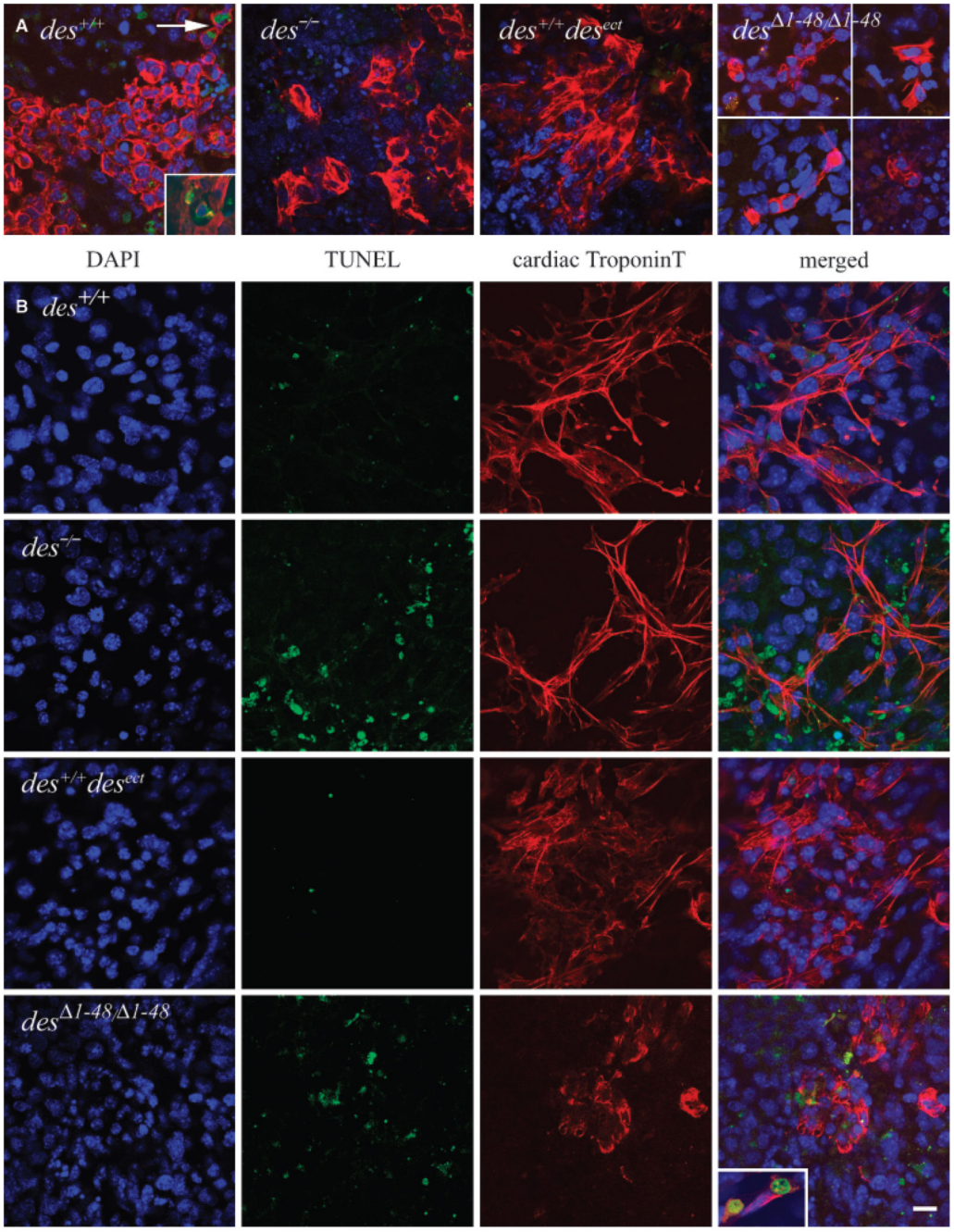

Fig. 5.

Apoptosis is absent in early differentiating cardiomyocytes but increases with age in *des*$^{-/-}$ and *desmin*$^{\Delta 1-48}$ expressing cardiomyocytes. Embryoid bodies (EBs) fixed on days 8 and 16 after embryonic stem cells aggregation, respectively, and indirectly stained with DAPI for nuclear DNA (blue), for single-strand brakes of DNA by TUNEL assay with fluorescein-labelled dNTPs (green), and for cTnT with anti-cTnT and secondary TRITC-conjugated antibodies (red). Genotypes as indicated. (**A**) Merged confocal triple immunofluorescence micrographs of typical cardiomyogenic areas in EBs at day 8. In the case of *des*$^{\Delta 1-48/\Delta 1-48}$

EBs images from different EBs were combined in order to show a larger number of the widely dispersed and rarely found cardiomyocytes. Arrow, two of 1,083 cardiomyocytes positive for TUNEL staining. Insets, examples of rarely found typical apoptotic cells with phosphatidylserine at the outer side of the plasma membranes. FITC-conjugated annexin V (green). (**B**) Cardiomyocytes in EBs at day 16. Note, that cTnT positive *des*$^{-/-}$ and *des*$^{\Delta 1-48/\Delta 1-48}$ cardiomyocytes are surrounded by TUNEL positive cells but very rarely are positive for TUNEL assay and cTnT (inset lower right panel). Scale bar, 25 μm.

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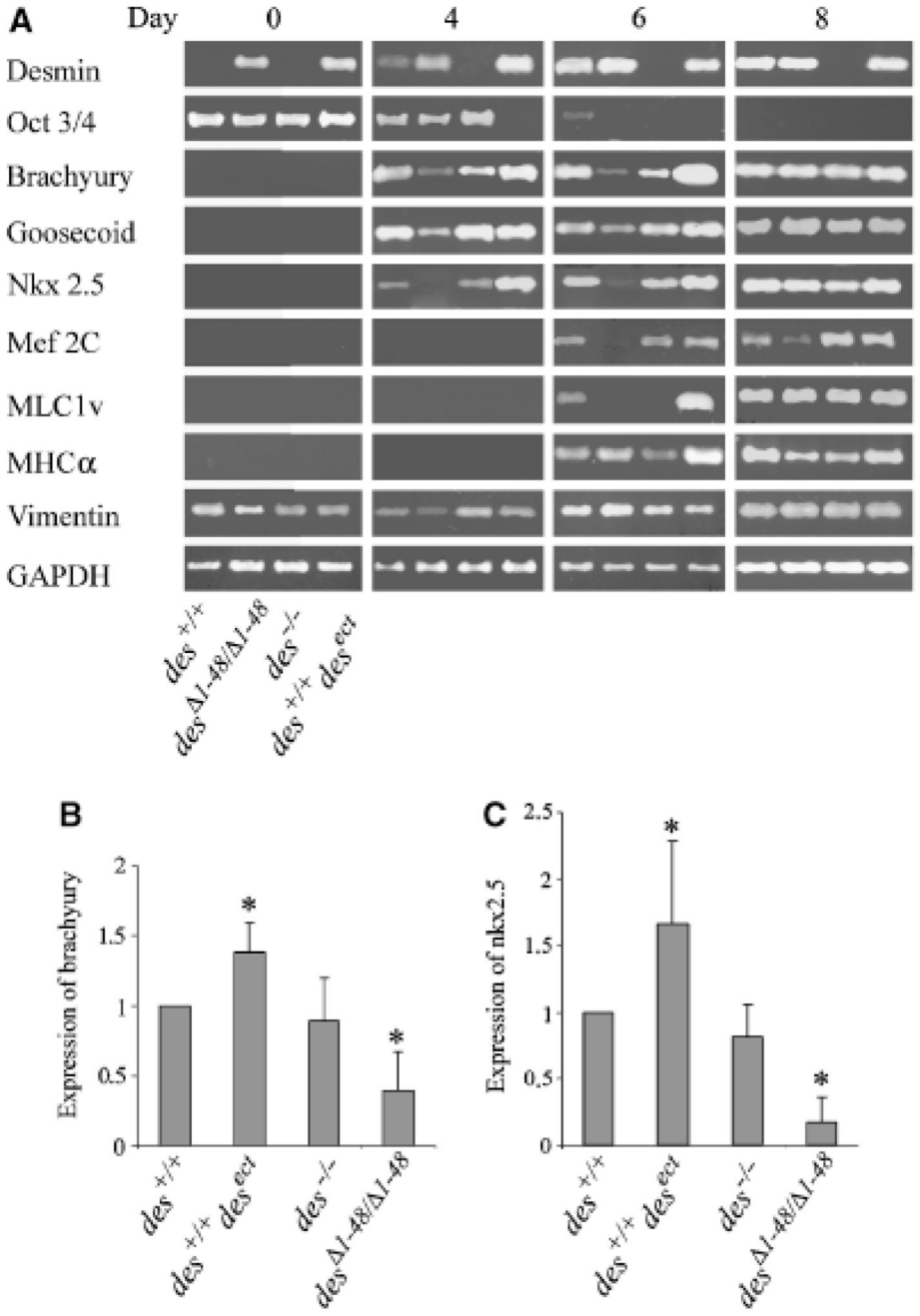

Fig. 6.

Premature synthesis of desmin causes increased expression of mesodermal and myocardial transcription factor genes in embryoid bodies (EBs). mRNA was isolated and reverse transcribed from embryonic stem cells (day 0) and EBs of genotypes as indicated in the lower left corner at days 4, 6, and 8, respectively. (**A**) Semiquantitative RT-PCR was performed in at least three independent experiments (one typical shown) with primer pairs as indicated. cDNA was normalized by levelling the GAPDH RT-PCR product. Statistical analysis of (**B**) *brachyury* and (**C**) *nkx2.5* expression in EBs between days 4 and 6 of

six independent experiments. Expression levels normalized to expression in wild-type EBs. *p-values smaller than 0.05 relate to control ($des^{+/+}$).

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Table 1

Mutant desmin increases cell death in embryoid bodies at the beginning of mesoderm formation

Genotype	% dead cells[1]	Standard deviation	p-value	Number of experiments
$des^{+/+}$	17.97	1.77	—	5
$des^{-/-}$	17.74	0.74	0.8123	5
$des^{+/+}des^{ect}$	19.85	1.03	0.1797	4
$des^{\Delta 1-48/\Delta 1-48}$	26.04	1.28	0.0008	5

[1] Cells of embryoid bodies were dissociated by mild digestion at day 5 and dying or dead cells were stained with propidium iodide and sorted by FACS analysis.