

Amino-terminally truncated desmin rescues fusion of $des^{-/-}$ myoblasts but negatively affects cardiomyogenesis and smooth muscle development

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Abstract Desmin fulfils important functions in maintenance of muscle cells and mutations in the *desmin* gene have been linked to a variety of myopathies. To ascertain the role of desmin's amino-terminal domain in muscle cells we generated embryonic stem cells constitutively expressing desmin $^{\Delta 1-48}$ in a null background and investigated muscle cell development in vitro. Desmin $^{\Delta 1-48}$ lacking the first 48 amino acid residues promotes fusion of myoblasts, rescues myogenesis and down-regulates vimentin expression in embryoid bodies, but hampers cardiomyogenesis and blocks smooth muscle development. These results demonstrate that desmin's amino-terminus has different roles in skeletal, cardiac, and smooth muscle cell development and function. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Desmin; Muscle development; In vitro differentiation; Cardiomyogenesis

1. Introduction

Acquired and congenital cardiomyopathies and myopathies are one of the major medical burdens. Reduced ventricular function correlates with cardiomyocyte degeneration, cellular atrophy and interstitial fibrosis. Apart from myofibril degeneration, cytoskeletal rearrangements are the most prominent primary and secondary events leading to end-stage heart diseases and myopathies [1].

The cytoskeleton in cardiomyocytes, skeletal, and smooth muscle cells is composed of the contractile apparatus, the sub-membrane skeleton of the nucleus and plasma membrane, including intercalated discs, costameres, and myotendinous junctions, and three groups of filaments, the microtubules, microfilaments, and intermediate filaments (IFs) [2]. The latter are composed of subunits expressed in a tissue-specific fashion, e.g. in muscle cells, desmin [3]. Desmin filaments provide a transcytoplasmic integrating matrix to striated muscle cells responsible for the alignment of sarcomeres [4], are essential for maintenance of muscle structure and function [5], render

possible proper cellular force transmission [6], and perhaps signal transduction. Defects in the desmin organisation have been linked to a variety of cardiomyopathies and skeletal muscle myopathies [7].

To fulfil these functions in muscle tissue desmin interacts with IF proteins, such as vimentin, paranemin, synemin, and nestin [3,8], with a variety of IF-associated proteins, and with components of the membrane skeleton, such as ankyrin to which desmin specifically binds via its amino-terminal head domain [9]. This domain, rich in serine residues phosphorylated by Rho kinase, p21-activated PAK kinase, cdc2 kinase, and protein kinases A and C [10], is a modulator of cell cycle- and development-dependent stability and function of IFs. Deletions within this domain altered the cellular organisation of desmin IFs [11]. Here we hypothesise that the amino-terminal domain of desmin fulfils specific roles in muscle cells and contributes to the interaction between cardiomyocytes and myocytes, respectively.

To demonstrate the different functions of the amino-terminal domain of desmin during embryonic muscle development we replaced both wild type alleles of the *desmin* gene in embryonic stem (ES) cells by the mutant allele *des* $^{\Delta 1-48}$. Desmin $^{\Delta 1-48}$ lacking amino acid residues 1–48 of the head domain is predominantly localised in the cytoplasm as dot-like structures and rarely co-assembled with pre-existing IFs. In *des* $^{\Delta 1-48/\Delta 1-48}$ embryoid bodies (EBs) fusion of polynucleated myotubes was partially restored; however, the *des* $^{-/-}$ smooth muscle phenotype could not be rescued. Cardiogenesis was significantly inhibited and cell–cell interaction and contraction was negatively affected, demonstrating that the amino-terminal domain of desmin has essential functions in cardiomyocyte, skeletal and smooth muscle development and maintenance of their phenotype.

2. Materials and methods

2.1. Vector construction and homologous recombination in ES cells

The replacement type vector contained a 6.5 kb *EcoRI* fragment of the murine *desmin* gene [12], linked to the herpes simplex virus thymidine kinase (*HSV-tk*) gene. The *neo* cassette [13] was inserted in 3' to 5' orientation between two *XhoI* sites in exon 1 allowing transcription of a mutated desmin message under the control of the *tk* promoter of the *neo* cassette. Culture, electroporation, and selection of AB2.1 ES cells on SNL76/7 fibroblasts in medium M15, as well as Southern blot, Northern blot, IF preparation, and Western blot analysis with monoclonal D3 anti-desmin antibodies (Hybridoma Bank, Ames, IA, USA) have been described previously [12,14,15]. Two out of 17 heterozygous cell lines were selected for gene conversion at the

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Abbreviations: ES cells, embryonic stem cells; EB, embryoid body; IF, intermediate filament; HSV, herpes simplex virus; tk, thymidine kinase

second allele resulting in seven homozygous cell lines with identical phenotype. Mutant desmin was immunoprecipitated with polyclonal desmin antibodies (Sigma #8281, 1:50) and detected with D3 antibodies. PCR and RT-PCR were performed with *desmin*-specific primers 5'-TGATGAGGCAGATGAGGGAG-3' and 5'-AACACCCTCTCTGTACCCTC-3', T_m 60°C, and a Py441 polyoma enhancer-specific primer 5'-GTAAGGTCCGGACCCACC-3' and *desmin*-specific primer 5'-ATAGGATGGCGCTCGGGT-3', T_m 49°C, respectively. Transfected SW13-IHF5 cells were selected for homologous recombination at 150 µg/ml G418. Chimeric mice were produced from four ES cell lines.

2.2. In vitro differentiation of ES cells and immunofluorescence microscopy

Each 400 ES cells were aggregated in 20 µl drops in M15 for 4 days, EBs were plated onto gelatinised (Difco) 10 cm tissue culture dishes and cardiomyogenesis, skeletal and smooth muscle development was monitored and analysed essentially as in [15].

Cells and EBs on gelatinised coverslips were washed with phosphate-buffered saline, fixed in 96% ethanol at -20°C for 20 min, stained for desmin with D3 and K5-pDes (Dr Gert Schaart, Universiteit Maastricht, The Netherlands, 1:25), connexin 43 (Sigma C6219, 1:400), vimentin (ICN 647401, 1:100), myosin MF20 (Hybridoma Bank), smooth muscle actin (Sigma #2547) or myogenin F5D antibodies (Dr Woody Wright, Pasteur Institute, Paris, France) for 90 min, and consecutively with FITC- and TRITC-conjugated secondary antibodies (Sigma F4018, 1:200; T7028, 1:200; T-5268, 1:80) for 90 min. Photomicrographs were taken on a Leica TCS SP confocal microscope.

3. Results

3.1. Expression of *desmin*^{Δ1-48} in *des*^{-/-} ES cells and embryoid bodies

ES cells expressing a mutated desmin protein lacking the amino-terminal membrane skeleton-binding domain in a desmin null background were generated by homologous recom-

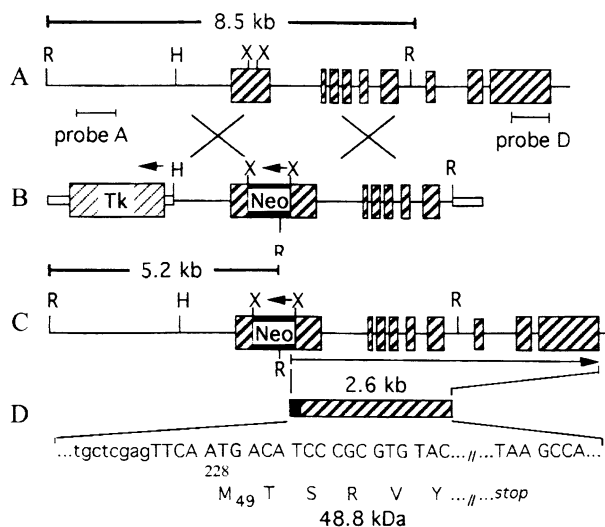


Fig. 1. Homologous recombination at the *desmin* locus replacing the wild type allele by *des*^{Δ1-48}. A: Restriction map of the murine *desmin* locus. B: Targeting vector. C: Mutant *des*^{Δ1-48} allele. Shaded boxes, exons; white boxes, cloning vector; Tk, HSV thymidine kinase gene; Neo, neomycin gene; arrows, orientation of *tk* promoters; bars, restriction fragments and probes used for identification of the mutant allele; R, *EcoRI*; H, *HindIII*; X, *XhoI*. D: mRNA transcribed from the *des*^{Δ1-48} allele. Note that the *tk* promoter in the *neo* cassette also drives transcription in the 5'-3' orientation of the locus (long arrow). The sequence shows the unique start codon at bp 228 in exon 1. Lower-case letters, *tk* promoter; upper-case letters, exon 1 of the *desmin* gene.

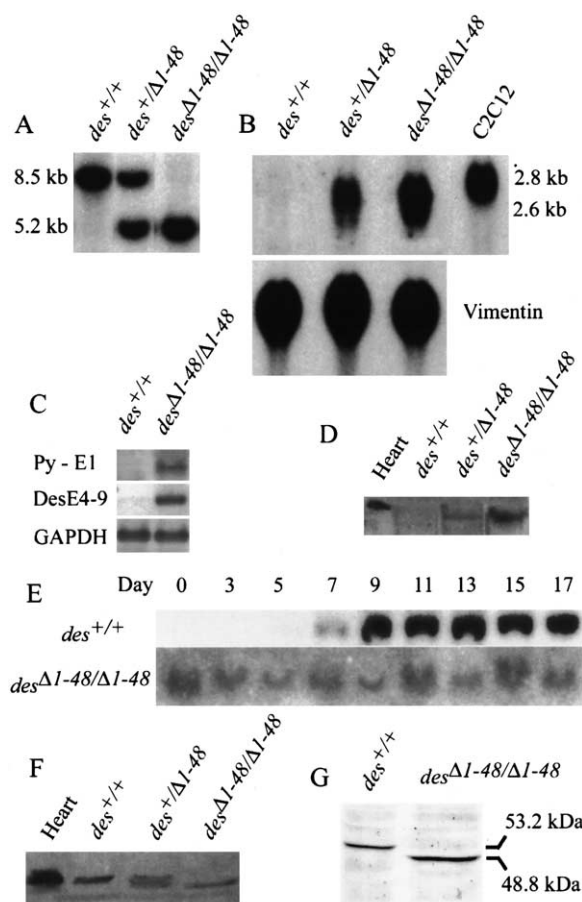


Fig. 2. Identification of the *des*^{Δ1-48} allele and desmin^{Δ1-48} protein in ES cells and EBs. A: Southern blot analysis of *EcoRI*-digested genomic DNA from *des*^{+/+}, *des*^{+/Δ1-48}, and *des*^{Δ1-48/Δ1-48} ES cell lines, hybridisation with probe A (see Fig. 1). B: Northern blot analysis of *des*^{+/+}, *des*^{+/Δ1-48}, and *des*^{Δ1-48/Δ1-48} undifferentiated ES cell lines, hybridisation with probe D (see Fig. 1). C2C12, positive control. C: RT-PCR with RNA from undifferentiated ES cells with primers amplifying the Py441 polyoma enhancer containing mutant exon 1 (Py-E1) fragment and exon 4 to exon 9, respectively. GAPDH, loading control. D: Western blot analysis of immunoprecipitated ES cell lysates. Heart, positive control. E: Northern blot analysis of RNA from developing EBs. F,G: Western blot analysis of IF preparations from 10 day old EBs (F) and from high resolution SDS-PAGE of desmin and desmin^{Δ1-48} (G).

bination at the *desmin* locus. A replacement type vector with the *MC1neo* cassette inversely inserted in front of the methionine 49 codon in exon 1 of the murine *desmin* gene was used to generate the *des*^{Δ1-48} allele (Fig. 1A–C). Spontaneous gene conversion resulted in seven *des*^{Δ1-48/Δ1-48} cell lines. Correct homologous recombination was verified with probes as indicated in Fig. 1 (Fig. 2A, and data not shown).

Constitutive transcription of a 2.6 kb mRNA in ES cells was exclusively driven by the weak *tk* promoter of the *MC1neo* cassette (Figs. 1D and 2B). RT-PCR with primers binding to the Py441 polyoma enhancer of the *MC1neo* cassette and the very 3' part of exon 1, and situated within exon 4 and the 3' untranslated region, respectively, demonstrated that mutated exons 1–9 were correctly and constitutively transcribed (Fig. 2C). Translation of desmin^{Δ1-48} starting at the bona fide start codon methionine 49 was verified by immunoprecipitation of ES cell lysates, and SDS-PAGE demonstrated a M_r of 48 800 close to the theoretical M_r of 48 386 (Fig.

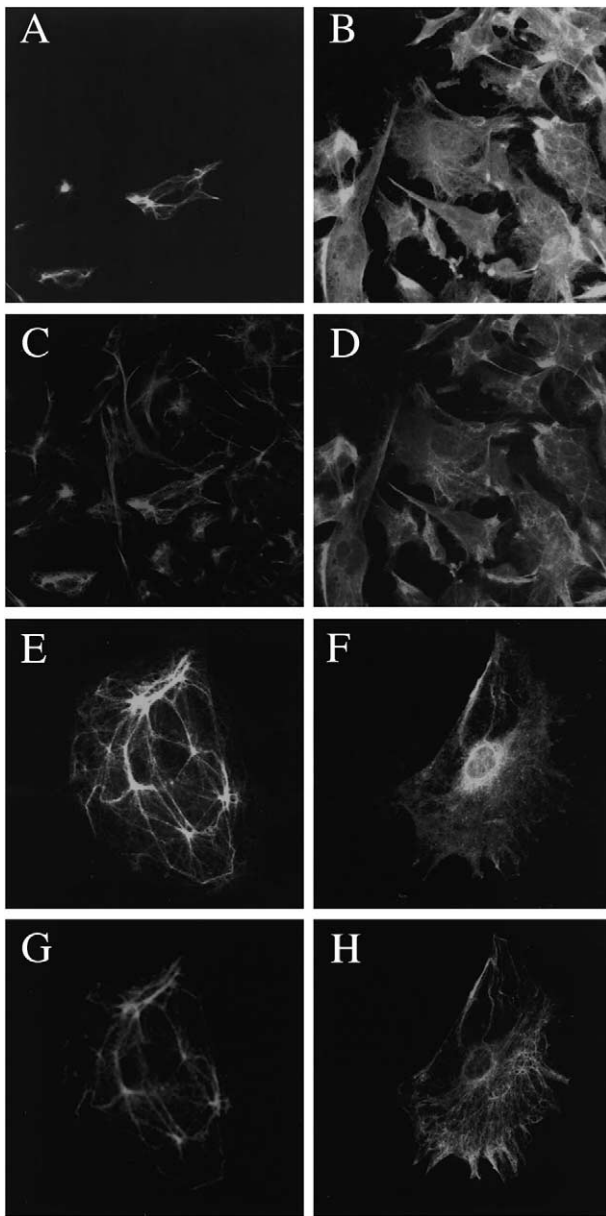


Fig. 3. Immunolocalisation of desmin, desmin $\Delta 1-48$ and vimentin in EBs, at day 13. Myoblasts and fibroblasts in wild type (A,C,E,G) and des $\Delta 1-48/\Delta 1-48$ EBs (B,D,F,H). A,B,E,F: Antibody to desmin. C,D,G,H: Antibody to vimentin. Magnification: (A–D) 400 \times , (E–H) 800 \times .

2D,G). In des $\Delta 1-48/\Delta 1-48$ EBs des $\Delta 1-48$ mRNA was transcribed throughout the period of in vitro development (Fig. 2E). Desmin $\Delta 1-48$ could be enriched in IF preparations of EBs (Fig. 2F), thus demonstrating that desmin $\Delta 1-48$ partially co-assembles with and perhaps influences other IFs.

Desmin $\Delta 1-48$ was identified in undifferentiated des $\Delta 1-48/\Delta 1-48$ ES cells as a weak and diffuse cytoplasmic staining (data not shown), and in all cells of des $\Delta 1-48/\Delta 1-48$ EBs (Fig. 3), whereas in wild type EBs only muscle cells expressed desmin [15]. Ectopically expressed desmin $\Delta 1-48$ was partially integrated in vimentin filaments, however, a significant part localised in the cytoplasm and perinuclear aggregates (Fig. 3F). In SW13-IHF5 adenocarcinoma cells lacking all IF proteins desmin $\Delta 1-48$ could not form de novo IFs, and caused cataract formation in the eye lens of chimeric mice (data not shown).

3.2. Expression of desmin $\Delta 1-48$ in a null background differentially affects heart, skeletal and smooth muscle development and vimentin expression in EBs

Development and function of des $\Delta 1-48/\Delta 1-48$ cardiomyocytes were severely compromised (Fig. 4A), whereas in des $^{+}/\Delta 1-48$ and des $^{-}/-$ EBs [15] no negative effect was observed. Rhythmic beating was decreased to 34 ± 11 min $^{-1}$, $n = 62$ (wild type: 91 ± 40 min $^{-1}$, $n = 84$), the number of beating foci per EB was reduced to 2.2 ± 1.1 (wild type: 4.3 ± 1.3) and the majority of foci was significantly smaller than in wild type EBs.

Skeletal muscle formation in EBs is initiated by fusion of presumptive myoblasts to small oligonucleated myotubes, followed by fusion to polynucleated contracting myotubes which finally form large arrays of well developed beating myotubes [15]. Constitutive expression of desmin $\Delta 1-48$ promoted development and/or fusion of presumptive myoblasts in a dose-dependent manner (Fig. 4B). Development of polynucleated contracting myotubes was partially rescued by desmin $\Delta 1-48$ (Fig. 4C). Myotubes expressed myosin (Fig. 4D) and myogenin (Fig. 4E), which was absent in des $^{-}/-$ myotubes [15], however, never formed large arrays over extended periods of time. Thus desmin $\Delta 1-48$ facilitates myogenic differentiation, effectively drives cells into fusion, but fails to sustain the adult skeletal muscle phenotype.

Development of contracting smooth muscle cells was significantly reduced in des $^{+}/\Delta 1-48$, and essentially absent in des $\Delta 1-48/\Delta 1-48$ EBs (Fig. 4F). The number of smooth muscle actin-positive fibroblasts was reduced (data not shown). These results suggest that desmin $\Delta 1-48$ cannot support the multistep transition process in which non-muscle cells are converted to myofibroblasts and to smooth muscle cells.

Vimentin expression continuously increased in wild type EBs and was up-regulated in des $^{-}/-$ EBs shortly after the time when desmin is expressed at the onset of cardiogenesis and myogenesis in wild type EBs (Figs. 2E and 4G). In des $\Delta 1-48/\Delta 1-48$ EBs increase in vimentin expression stopped at the time when cardiomyocytes and myoblasts started to develop (Fig. 4G). These results demonstrate that desmin $\Delta 1-48$ influences vimentin expression and suggest that vimentin may step in for desmin in des $^{-}/-$ EBs but not in EBs with functionally compromised desmin.

4. Discussion

A common feature of IF proteins is that their amino-terminal domain interacts with a variety of cellular targets and contributes to their tissue-specific function [3,16]. Knock-out of the desmin gene demonstrated its significant roles in cardiac, skeletal and smooth muscle function [5,17,18]. Here we reported that desmin $\Delta 1-48$ lacking 48 amino acids of the amino-terminal head domain affects cardiac, skeletal and smooth muscle to different degrees in EBs thus demonstrating that desmin exerts different roles via its head domain during muscle development.

Stable but low expression of desmin $\Delta 1-48$ in a null background in ES cells and EBs was achieved by introduction of a reverted HSV-*tk* promoter in front of the methionine 49 codon in exon 1 of both alleles of the murine desmin gene. High level expression of desmin or mutated desmin was toxic to ES cells (Puz and Weitzer, unpublished results). Constitutive transcription of a single, correctly spliced mRNA, exclud-

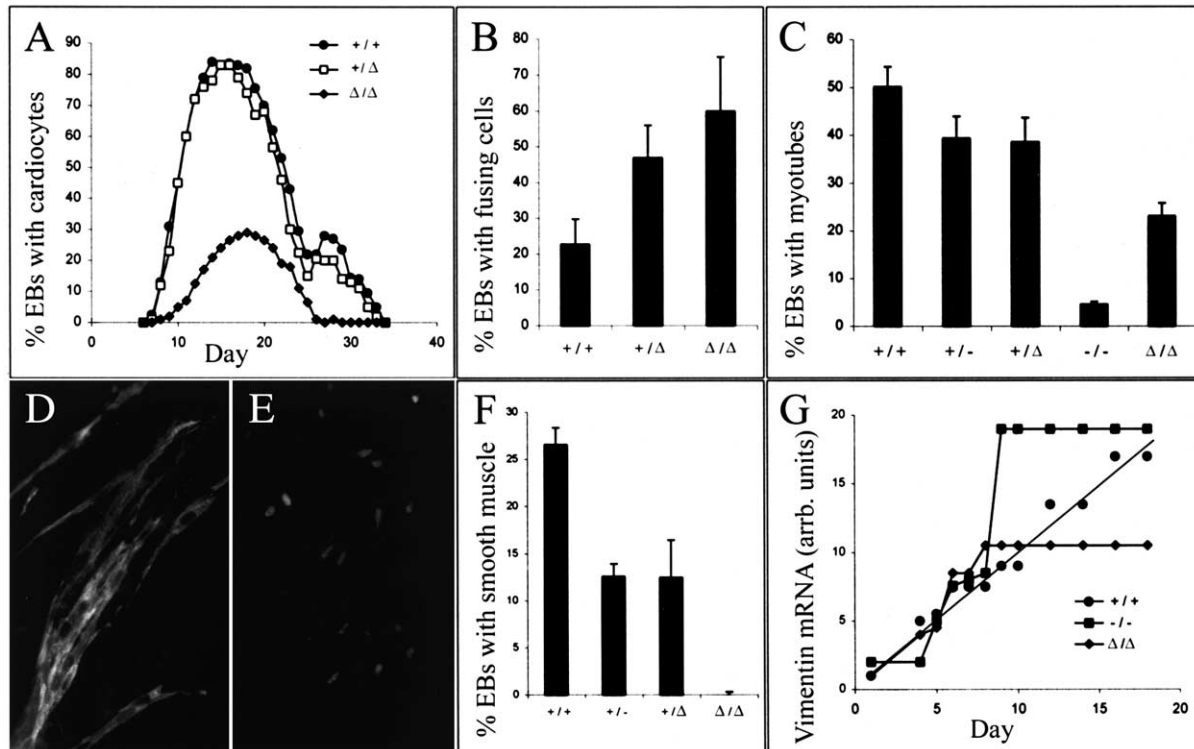


Fig. 4. Influence of desmin $\Delta 1-48$ on cardiomyogenesis, skeletal and smooth muscle myogenesis in a $des^{-/-}$ background. A: Development of beating cardiomyocytes in $des^{+/+}$ (●, +/+), $des^{+/\Delta 1-48}$ (□, +/-), and $des^{\Delta 1-48/\Delta 1-48}$ (◆, Δ/Δ) EBs. Number of cell lines and EBs checked: $des^{+/+}$, 2/5990; $des^{+/\Delta 1-48}$, 5/3045; $des^{\Delta 1-48/\Delta 1-48}$, 4/6806. B,C: Percentage EBs with fusing cells (B) and contracting myotubes (C). $des^{+/+}$, (+/-); $des^{-/-}$, (-/-). Mean values were calculated from maxima ± 2 days; $des^{+/+}$, 2/3471; $des^{+/-}$, 5/4502; $des^{+/\Delta 1-48}$, 5/3694; $des^{-/-}$, 7/3665; $des^{\Delta 1-48/\Delta 1-48}$, 4/4244. D,E: Myosin (D) and myogenin immunolocalisation (E) in $des^{\Delta 1-48/\Delta 1-48}$ myotubes. Magnification 400 \times . F: Percentage EBs with contracting smooth muscle cells. $des^{+/+}$, 2/1004; $des^{+/-}$, 5/4502; $des^{+/\Delta 1-48}$, 5/3694; $des^{\Delta 1-48/\Delta 1-48}$, 4/6501. G: Expression of vimentin mRNA in EBs. Each 20 μ g mRNA from $des^{+/+}$ (●), $des^{\Delta 1-48/\Delta 1-48}$ (◆), and $des^{-/-}$ (■) EBs were analysed by semi-quantitative Northern blotting.

ing dicistronic expression, gave rise to translation of desmin $\Delta 1-48$. Desmin $\Delta 1-48$ could not form de novo filaments or aggregates in ES or SW13-IHF5 cells, partially integrated in pre-existing IFs in differentiated cell types and was also found as perinuclear aggregates as observed in several desmin-related cardiomyopathies [7]. Desmin $\Delta 1-48$ had no obvious influence on any other cell type than muscle cells because development of extra-embryonic endoderm, neurones and erythrocytes was not affected in $des^{\Delta 1-48/\Delta 1-48}$ EBs. Ectopic expression of desmin $\Delta 1-48$ was demonstrated in eye lenses of chimeric mice, however, germline transmission of the $des^{\Delta 1-48}$ allele was never possible due to severe malformation of male and female reproductive organs, phenotypically resembling hermaphrodites. All mice with more than 30% of the coat coloured died within weeks suggesting that desmin $\Delta 1-48$ expression was lethal.

In EBs desmin $\Delta 1-48$ differentially influenced the development of cardiac, skeletal and smooth muscle cells. In $des^{+/\Delta 1-48}$ EBs cardiomyocytes were not affected, smooth muscle cells were negatively affected, and fusion of myoblasts was promoted. In $des^{\Delta 1-48/\Delta 1-48}$ EBs cardiomyogenesis was severely hampered. Reduced numbers of cardiomyocytes per beating focus and significantly reduced beating rates, which were not observed in $des^{-/-}$ EBs [15], and reduced expression of connexin 43 in $des^{\Delta 1-48/\Delta 1-48}$ cardiomyocytes (data not shown) suggest that headless desmin actively interferes with

either cell–cell recognition, attachment or signal transduction between cardiomyocytes.

Smooth muscle cells were essentially absent in desmin $\Delta 1-48$ EBs and the number of myofibroblasts, supposed to be intermediates in smooth muscle development, was reduced. These results correlate with the requirement of desmin in small artery smooth muscle cells [18], and indirectly suggest an important function of desmin's head domain in smooth muscle development; however, the mechanism remains elusive.

Fusion of presumptive skeletal muscle myoblasts was significantly increased, thus desmin $\Delta 1-48$ rescued the $des^{-/-}$ phenotype [15] either by increasing the number of early myoblasts and/or by promoting fusion of non-myogenic cells. The discrepancy between the in vitro (this study and [15]) and in vivo data [19] may well arise from different environmental influences in EBs and somites, respectively. Absence of extracellular matrix or paracrine signals may cause indispensability of desmin in EBs. Timely onset of fusion demonstrates that in vitro desmin is an essential, but not the committing factor in myoblasts, and that non-filamentous desmin $\Delta 1-48$ is sufficient for cell fusion. However, the absence of large long-living myotubes in $des^{\Delta 1-48/\Delta 1-48}$ EBs suggests that desmin's head domain is indispensable for maintenance of the skeletal muscle phenotype. Down-regulation of vimentin expression by desmin $\Delta 1-48$ may in addition be responsible for the reduced longevity of both skeletal muscle cells and cardiomyocytes. On

the other hand, up-regulation of vimentin in *des*^{-/-} EBs might sustain cardiomyogenesis but not skeletal muscle development, because vimentin cannot drive fusion. Here we demonstrated for the first time that desmin and vimentin expression is linked by a feedback loop, thus providing evidence for the hypothesis that desmin might be directly or indirectly, via diverting differentiation, involved in signal transduction modulating the expression of other IF proteins.

Desmin is not necessary for embryogenesis [19,20]; however, absence of desmin causes severe defects in all muscle tissues resembling the pathology of myopathies and cardiomyopathies in the adult [5,17,18]. Additionally, mutant *desmin* alleles have been linked to various cardiomyopathies [1] and here we suggest that mutations or deletions within the amino-terminal domain of desmin may also contribute to cardiomyopathies. Finally, these in vitro experiments made it possible to assess the differential influence of *desmin*^{Δ1–48} on heart, skeletal and smooth muscle cell development which was not possible in vivo, due to lack of germline transmission of the *des*^{Δ1–48} allele.

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