Disruption of Muscle Architecture and Myocardial Degeneration in Mice Lacking Desmin

Derek J. Milner, Georg Weitzer, Duyen Tran, Allan Bradley,* and Yassemi Capetanaki

Department of Cell Biology and *Howard Hughes Medical Institute and Institute for Molecular Genetics, Baylor College of Medicine, Houston, Texas 77030

Abstract. Desmin, the muscle specific intermediate filament (IF) protein encoded by a single gene, is expressed in all muscle tissues. In mature striated muscle, desmin IFs surround the Z-discs, interlink them together and integrate the contractile apparatus with the sarcolemma and the nucleus. To investigate the function of desmin in all three muscle types in vivo, we generated desmin null mice through homologous recombination. Surprisingly, desmin null mice are viable and fertile. However, these mice demonstrated a multisystem disorder involving cardiac, skeletal, and smooth muscle. Histological and electron microscopic analysis in both heart and skeletal muscle tissues revealed severe disruption of muscle architecture and degeneration. Structural abnormalities included loss of lateral alignment of myofibrils and abnormal mitochondrial organization. The consequences of these abnormalities were most severe in the heart, which exhibited progressive degeneration and necrosis of the myocardium accompanied by extensive calcification. Abnormalities of smooth muscle included hypoplasia and degeneration. The present data demonstrate the essential role of desmin in the maintenance of myofibril, myofiber, and whole muscle tissue structural and functional integrity, and show that the absence of desmin leads to muscle degeneration.

THE intermediate filaments (IFs)[1] form one of the three cytoskeletal networks found in higher eukaryotes. Unlike microtubules and microfilaments, which are built from subunits that are ubiquitously expressed, the proteins which form IFs display a very tissue specific and developmentally regulated pattern of expression (for review see Lazarides, 1980; Steinert and Roop, 1988; Fuchs and Weber, 1994). Numerous plausible functions for IFs have been proposed over the years, including functioning as strength supporters and mechanical integrators of intracellular space, influencing of cell shape, providing of a mechanical scaffold for biochemical reactions, involvement in intracellular transport, in regulation of gene expression, and in signal transduction (for review see Fuchs, 1994; Traub and Shoeman, 1994; Evans, 1994; Klymkowsky, 1995). However, a well-defined function for some IFs, including desmin, the muscle specific IF protein (Lazarides and Hubbard, 1976), remains elusive.

Desmin is encoded by a single gene (Capetanaki et al., 1984) and is expressed in all muscle tissue (for review see Lazarides, 1980; Lazarides and Capetanaki, 1986). It is one of the earliest known myogenic markers to be expressed in both cardiac and skeletal muscle (Holtzer et al., 1982; Kaufmann and Foster, 1988; Herrmann et al., 1989; Schaart et al., 1989; Choi et al., 1990; Mayo et al., 1992). Desmin first appears at day 8.25 post coitum (pc) in the neuroectoderm, where it is transiently expressed together with vimentin, nestin, and keratin. At day 8.5 pc the protein can be detected in the heart rudiment, and at day 9 pc, in the somites where it is coexpressed with vimentin and nestin (Herrmann et al., 1989; Schaart et al., 1989; Choi et al., 1990; Mayo et al., 1992; Sejernsen and Lendahl, 1993; Kachinsky et al., 1994, 1995). During skeletal muscle differentiation desmin expression precedes not only that of all muscle specific structural proteins examined (Lin et al., 1994), but also all of the members of the myoD family with the exception of myf5 (Arnold and Braun, 1993; Sassoon, 1993; Lyons and Buckingham, 1992, 1993; Smith et al., 1994), which is most possibly responsible for the initial activation of the desmin gene (Li and Capetanaki, 1993, 1994; Kuisk et al., 1996). In contrast to most muscle specific genes, desmin is also expressed at low levels in satellite cells and replicating myoblasts together with vimentin (Allen et al., 1991; Kaufmann and Foster, 1988). Terminal differentiation of skeletal muscle is accompanied by down-regulation of vimentin and accumulation of desmin (Bennett et al., 1979; Gard and Lazarides, 1980; Capetanaki et al., 1984*a*,*b*; Olson and Capetanaki, 1989). During the late stages of this process IFs gradually rearrange and assem-

Please address all correspondence to Y. Capetanaki, Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030. Tel.: (713) 798-4609. Fax: (713) 798-8005.

The current address of G. Weitzer is Institute for Biochemistry, University of Vienna, Vienna, Austria.

1. *Abbreviations used in this paper*: ES, embryonic stem; IF, intermediate filament; pc, post coitum.

ble at the peripheries of the Z-disks (Bennett et al., 1979; Gard and Lazarides, 1980; Tokuyasu et al., 1984). In all mature striated muscle, desmin IFs seem to link individual myofibrils laterally to each other at their Z-disks and to other intracellular structures, including the costameres and intercalated discs of the sarcolemma, mitochondria, T-tubules, and the nucleus (Granger and Lazarides, 1978, 1979; Bennett et al., 1979; Richardson et al., 1981; Tokuyasu et al., 1983*a,b*; Nelson and Lazarides, 1984; Danto and Fischman, 1984; Thornell et al., 1985; Lockhardt and Bloom, 1993; for review see Lazarides and Capetanaki, 1986; Small et al., 1992; Price, 1992; Georgatos and Maison, 1996).

The fact that desmin is expressed very early in muscle development, preceding all but one of the myogenic bHLH factors and all muscle structural proteins examined to date, suggests that it may play some sort of modulating role during myogenic commitment and differentiation. Indeed, studies done in vitro have provided some support to this hypothesis. Microinjection of antibodies specific to kinase A sites of desmin not only block the phosphorylation of desmin at these sites, but also block the ability of microinjected cells to fuse and form myotubes (Tao and Ip, 1991). Inhibition of desmin expression in C_2C_{12} myoblasts using antisense RNA technology blocked myotube formation and perturbed the expression of myoD and myogenin in these cells (Li et al., 1994). Additionally, recent studies with the in vitro differentiation of mouse embryonic stem (ES) cells in which one or both copies of the desmin gene were rendered inactive by homologous recombination gave further support to this possibility. While wild-type ES cells could form all three types of muscle cells during differentiation in vitro, ES cells with both copies of the desmin gene inactivated could not form smooth or skeletal muscle when allowed to differentiate (Weitzer et al., 1995). These experiments suggest the possibility that during early embryonic development desmin might play a critical role either in muscle differentiation and morphogenesis, potentially by supporting signal transduciton, as has been previously proposed (Wang et al., 1993; Ingber, 1993; Li et al., 1994; Weitzer et al., 1995; Forgacs, 1995), or/and in muscle maintenance. They further predict that the absence of desmin during embryonic development in vivo might result in defects in muscle formation and/or integrity and maintenance, depending on the ability of the other IF proteins expressed in early development, including at least vimentin and nestin (Sejernsen and Lendahl, 1993; Kachinsky et al., 1994, 1995), to compensate for desmin's absence.

Regardless of the many potential functions IFs may fulfill, it is becoming increasingly clear that they are crucial for providing and maintaining strength and mechanical integrity for the cell (Bonifas et al., 1991; Coulombe et al., 1991*a,b*; Cheng et al., 1992; Chipev et al., 1992; Lane et al., 1992; Rothnagel et al., 1992; Cote et al., 1993; Xu et al., 1993; Lee and Cleveland, 1994; Collard et al., 1995; for review, see Fuchs, 1994; Lee and Cleveland, 1994; McLean and Lane, 1995). The normal development and proper functioning of a multicellular organism depends upon the maintenance of structural and functional integrity. Disruption of this integrity will lead to tissue degeneration, the hallmark of several human diseases including several muscular and neuronal disorders. Specifically, several epidermal and neuronal disorders have been linked to aberrant organization of the intermediate filament (IF) cytoskeleton (for review see Fuchs, 1994; Lee and Cleveland, 1994; McLean and Lane, 1995). Similarly, overexpression of vimentin or desmin in the lens has been shown to lead to cataract formation and lens cell death (Capetanaki et al., 1989; Dunia et al., 1990). There are now several reports of cardiomyopathies and skeletal myopathies that are associated with abnormal accumulation or organization of desmin (Thornell et al., 1980, 1983; Vajsar et al., 1993; Horowitz and Schmalbruch, 1994; Ariza et al., 1995; for review see Goebel and Bornemann, 1993). Thus, in mature muscle, desmin might be crucial for maintaining the mechanical integrity and strength of myofibers.

To further define the function of desmin in vivo, we used homologous recombination to generate mice lacking desmin. Surprisingly, desmin null mice are viable and develop functioning cardiac, skeletal and smooth muscle, demonstrating that desmin is not necessary for muscle formation in vivo. However, they show defects in all three types of muscle tissue. Common features in all types of muscle include overt cell and tissue damage with signs of instability and disintegration, although only a fraction of muscle fibers in any tissue show defects. Histological and electron microscopic analysis of striated muscle tissue reveals that a fraction of myofibers display severe disruption of muscle architecture and signs of degeneration. The consequences of these abnormalities are most severe in the heart, which exhibits progressive degeneration and necrosis of the myocardium accompanied by extensive calcification and fibrosis. The data presented here demonstrate the importance of desmin in the maintenance of myofibril, myofiber, and whole muscle tissue structural and functional integrity, and show that the absence of desmin leads to muscle degeneration.

Materials and Methods

Construction of Targeting Vectors and Disruption of the Desmin Gene by Homologous Recombination

Details of the construction of the desmin targeting vector (see Fig. 1) and production of targeted ES cell lines for microinjection have been described previously (Weitzer et al., 1995). A fragment of DNA containing exons 1 to 6 of the desmin gene was ligated to a thymidine kinase (tk) expression cassette at its 5′ end and cloned into pBluescriptKS- (Stratagene, La Jolla, CA). The neo cassette from pMCIneopA (Thomas and Capecchi, 1987) was excised with XhoI and SalI and inserted between two XhoI sites located 107 and 142 bp downstream from the start codon in exon 1 (Li et al., 1994). Both marker cassettes were oriented in the opposite direction of the desmin sense strand. The 3′ part of exon 1 (basepairs 142 to 607 [SalI site]), downstream of the inserted neo cassette was removed to delete an internal start codon. Microinjections and other basic techniques were carried out essentially as described previously (Robertson, 1987).

Southern, Northern, and Western Blot Analysis

All blotting experiments were carried out essentially as described previously (Maniatis et al., 1982). Genomic DNA for genotyping was isolated from tail tips of 3–4-wk-old mice and digested with EcoRI. For identification of wild-type and targeted desmin alleles, a fragment from the 5′ flanking region of the desmin allele ("probe A," see Fig. 1 *A*) was isolated and used to generate ^{32}P-labeled probes by random priming. For Northern analysis, total RNA was isolated from skeletal muscle using guanidinium thiocyanate and phenol/chloroform, and blots were probed with ^{32}P-labeled

probes generated from cDNAs encoding mouse desmin or vimentin. For Western blotting, IF preparations were made from 200 mg of skeletal muscle by grinding fresh tissue under liquid nitrogen followed by extraction with PBS containing 0.6 M KCl, 1% Triton X-100, 1 mM PMSF and 1 mg/ml N_α-*p*-tosyl-arginine methyl ester (TAME) on ice. The resulting IF containing pellets were suspended in SDS-PAGE loading buffer and boiled. Proteins transferred to nitrocellulose were probed for desmin using monoclonal antibody D3 or rabbit polyclonal anti-desmin (Sigma Chem. Co., St. Louis, MO), and probed for vimentin using a goat polyclonal anti-vimentin (ICN Biomedicals, Costa Mesa, CA). Immunodetection was performed with the ECL system (Amersham Corp., Arlington Heights, IL).

Immunofluorescence

Sections of tissue were dissected from mice, rinsed in 1× PBS, immersed in OCT compound (Miles Inc., Elkhart, IN), and snap frozen in a dry ice/ethanol bath or liquid nitrogen. Tissue sections were cut at a thickness of ~5 μm and either used immediately for immunostaining or stored for a maximum of one week before use. For immunostaining, tissue sections were first rinsed briefly in 1× PBS and fixed in 100% ethanol for 10 min at −20°C. After three 5-min washes in 1× PBS, tissue sections were blocked by incubation in Blotto (1× PBS containing 5% powdered milk and 0.02% sodium azide) for at least 1 h at room temperature. The sections were then incubated with primary antibodies diluted in Blotto overnight in a humid chamber at 4°C. The sections were then rinsed extensively with 1× PBS and incubated with fluorochrome-conjugated secondary antibodies diluted in Blotto for 1–2 h in a humid chamber at room temperature. After extensive rinsing with 1× PBS, tissue sections were mounted in permafluor mounting media (Lipshaw Immunon, Pittsburgh, PA). Sections were observed on a Zeiss axiophot microscope and images were acquired using a Hamamatsu CCD camera. Staining for desmin was carried out using monoclonal antibodies DE-U-10 (Sigma) and D3 (Developmental

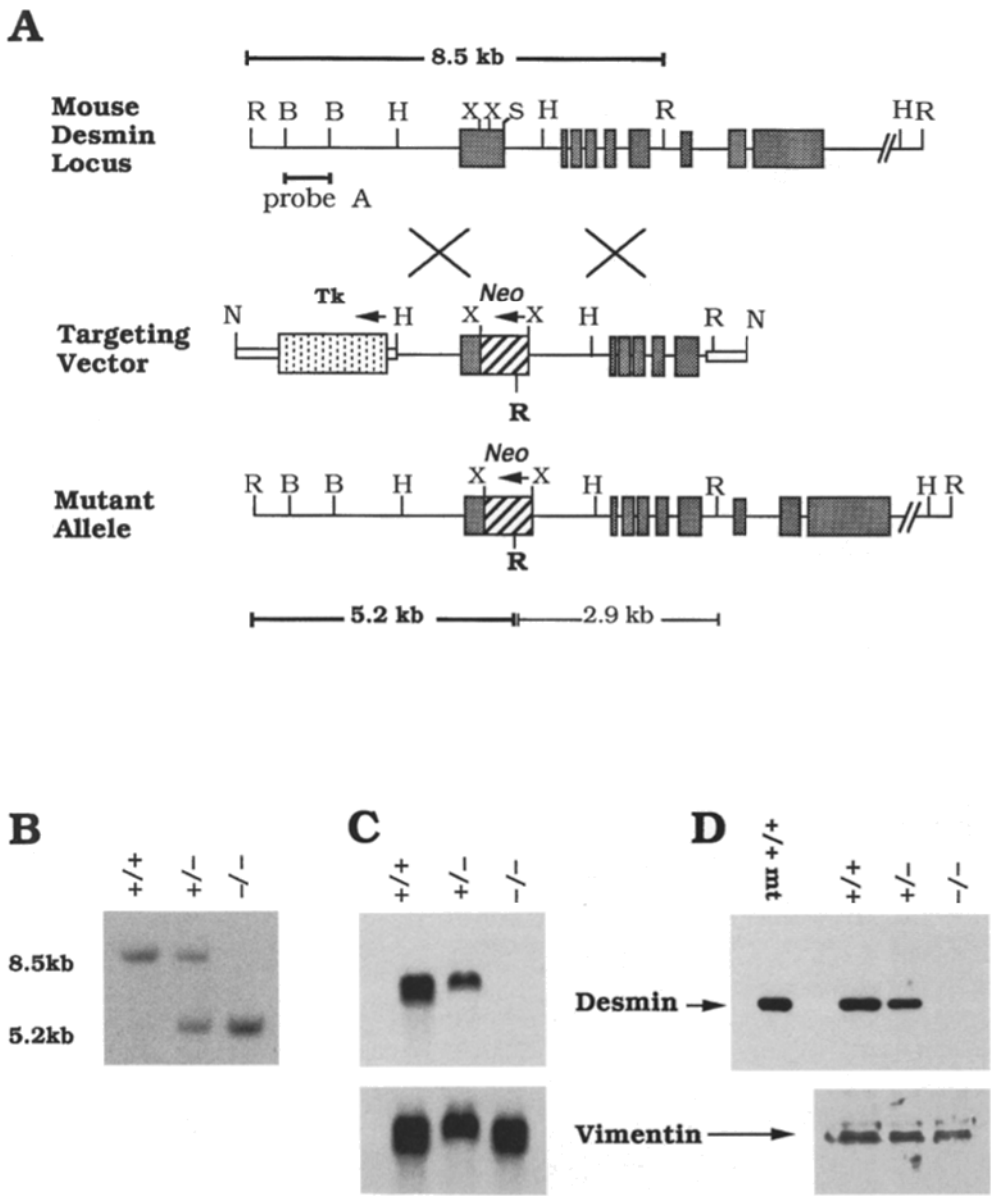


Figure 1. Targeted inactivation of the desmin gene. (*A*) Schematic representation of the mouse desmin locus, the targeting vector, and the mutant allele (des^{ml}) produced by homologous recombination. The nine exons of the mouse desmin gene are indicated by stippled boxes. The neo expression cassette disrupts the desmin coding sequence by replacing the sequence 3′ to the first XhoI site of exon 1. The EcoRI site in the neo expression cassette provides a marker for identifying targeted alleles. Digestion of genomic DNA with EcoRI produces a 5.2-kb fragment from the targeted allele compared to the 8.5-kb fragment produced from the wild-type allele. *R*, EcoRI; *B*, BamHI; *H*, HindIII; *X*, XhoI; *S*, SalI; *N*, NotI. (*B*) Southern blot analysis of EcoRI digested genomic DNA from wild-type $des^{+/+}$ (+/+), heterozygous $des^{+/ml}$ (+/−) and desmin null $des^{ml/ml}$ (−/−) mice. The indicated probe A was used for genotyping. (*C*) Northern blot analysis of skeletal muscle RNA isolated from 5-wk-old $des^{+/+}$, $des^{+/ml}$, and $des^{ml/ml}$ mice probed with a mouse desmin cDNA. No hybridization signal is detectable in $des^{ml/ml}$ RNA, even after overexposure (not shown). The bottom panel shows the same blot probed with a cDNA for vimentin, demonstrating comparable RNA loading and lack of change in vimentin expression levels. Note that the heterozygous lane is slightly underloaded. (*D*) Western analysis of intermediate filament (IF) preparations from skeletal muscle isolated from $des^{+/+}$, $des^{+/ml}$, and $des^{ml/ml}$ mice. +/+mt: IF preparation from C_2C_{12} myotube cell culture. The blot was probed with the monoclonal desmin antibody, D3. No desmin immunoreactivity is observed in the $des^{ml/ml}$ lane. The same results are obtained when the blot is probed with a polyclonal desmin antibody (not shown). The bottom panel shows a blot probed for vimentin. Note that there is no apparent overexpression of vimentin protein to compensate for the absence of desmin.

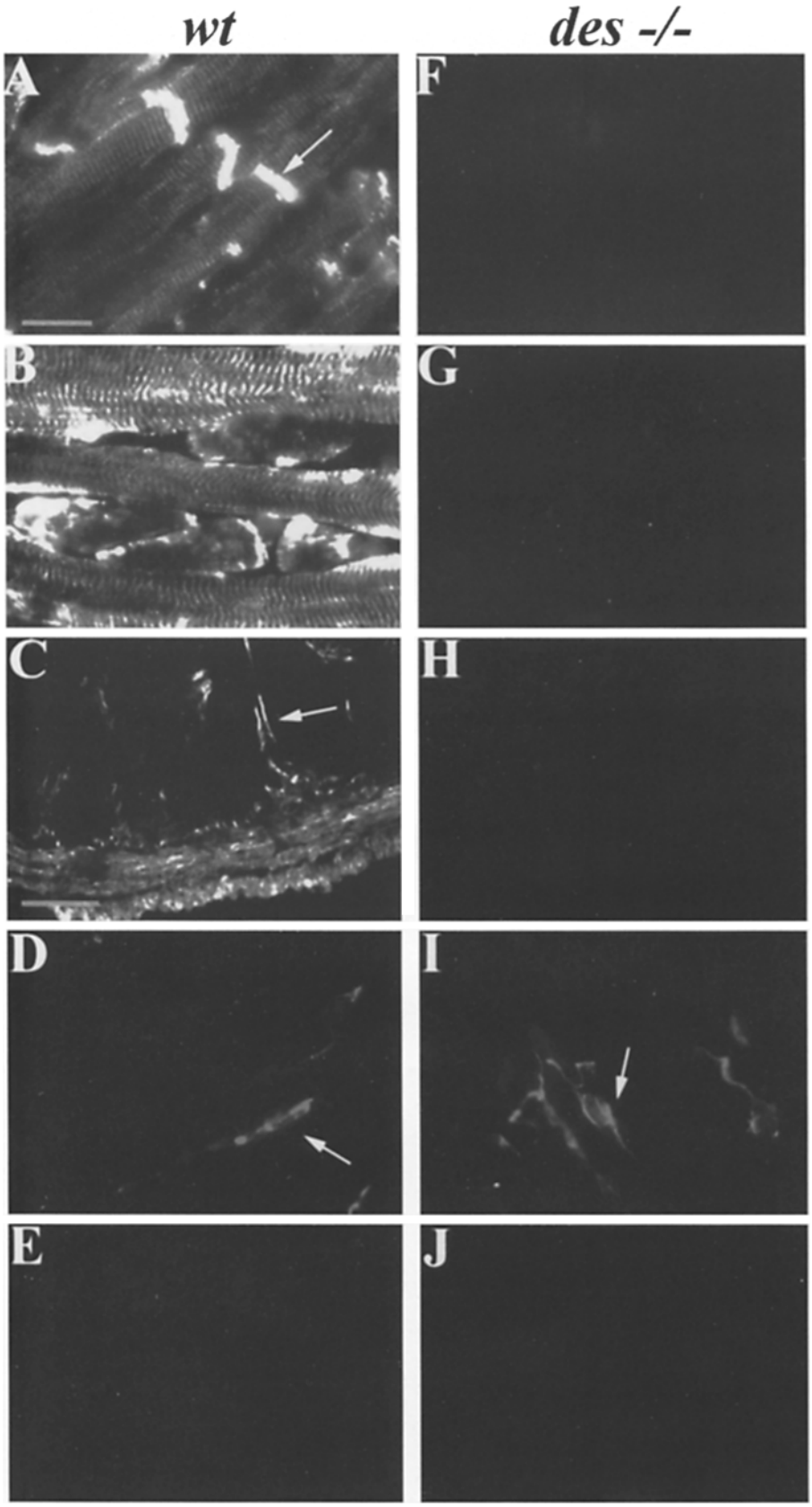


Figure 2. Analysis of intermediate filament protein expression by immunofluorescence. Frozen sections from cardiac muscle (*A* and *F*), skeletal muscle from the tongue (*B* and *G*), and smooth muscle (*C* and *H*) from the gut were stained using a desmin monoclonal antibody, DE-U-10. Desmin staining is completely absent from all muscle tissues from mice lacking desmin, while wild-type muscle tissue shows normal desmin-staining patterns. Skeletal and cardiac muscle show staining at the Z-discs, with prominent staining at the intercalated discs of cardiac muscle (*arrow*, *A*). Staining is seen in the smooth muscle of the wild-type gut, including in individual myofibers extending up into the villi (*arrow*, *C*). Frozen sections from the tongue (*D*, *E*, *I*, and *J*) were also stained for vimentin (*D* and *I*) and nestin (*E* and *J*). Vimentin staining is seen in individual fibroblasts and satellite cells (*arrows*, *D* and *I*), but is absent in myofibers from both wild-type (*D*) and null (*I*) muscle. Nestin staining is negative in both wild-type (*E*) and null (*J*) muscle as well. Bars: (*A*, *B*, and *D–G*, *I*, and *J*) 25 μm; (*C* and *H*) 40 μm.

Studies Hybridoma Bank [DSHB], Iowa City, IA), and rabbit polyclonal anti-desmin (Sigma D-8281). Vimentin staining was carried out with polyclonal anti-mouse vimentin from ICN Biomedicals, and nestin staining was performed using monoclonal antibody Rat 401 (DSHB). Monoclonal antibody MF20 (DSHB) was used for the staining of sarcomeric myosin heavy chain. Monoclonal antibodies for nebulin, α-actinin, tropomyosin, and smooth muscle α-actin were obtained from Sigma. Secondary antibodies used were FITC-conjugated goat anti-mouse IgG and Texas red–conjugated anti-rabbit IgG (Cappel, Malvern, PA) and rhodamine-conjugated anti-goat IgG (ICN). All antibody dilutions used were suggested by the manufacturer, with the exception of D3 and Rat 401, which were used as undiluted hybridoma supernatants.

Histology and Electron Microscopy

All tissues used for routine histological analysis were first fixed in Bouin's fixative overnight, and then embedded in paraffin, cut in 5-micron-thick sections and stained with hematoxylin and eosin. VonKossa staining was performed on frozen tissue sections fixed in a solution of formalin and ethanol. For staining with Masson's trichrome, tissues were fixed in 1× PBS containing 2% paraformaldehyde and 2% glutaraldehyde for 1 h at 4°C, followed by fixation in 10% neutral buffered formalin overnight. For electron microscopy, muscle tissue was fixed in glutaraldehyde, postfixed in osmium tetroxide, and embedded in Spurr's resin. 80-nm-thick sections were contrasted with lead and uranyl salts and photographed on an EM410 electron microscope from Philips Electronic Instruments (Eindhoven, The Netherlands).

Results

Generation of Desmin Null Mice

To generate mice lacking desmin, we used gene targeting via homologous recombination. The replacement vector used to generate the targeted disruption/deletion mutation (des^{ml}) at the first exon of the desmin gene as shown in Fig. 1 was described in detail recently (Weitzer et al., 1995). The 319-bp of the 3′ end of exon one was deleted to exclude the possibility of producing a partially functioning allele. This was done because we had initially found that transcription of this region governed by the HSV tk promoter produced a stable mRNA, and this transcript could produce a biologically active, truncated desmin polypeptide initiated from a bona fide downstream ATG codon. Targeted AB2.2 ES cell lines were microinjected into C57Bl/6 blastocysts and subsequently transferred into pseudopregnant females. The four different targeted cell lines used for microinjection yielded chimeras ranging from 20 to 80% ES cell contribution as estimated by coat color mosaicism. Chimeras were bred to C57Bl/6 females and germline transmission of the ES cell genome was obtained as verified by the production of agouti offspring. These mice were genotyped to identify mice heterozygous for the disrupted desmin allele ($des^{+/ml}$) using a probe generated from the 5′ flanking region of the mouse desmin locus, probe A (Fig. 1 *A*). Hybridization of this band to EcoRI digested genomic DNA identifies an 8.5-kb band from the wild-type desmin allele and a 5.2-kb band from the disrupted desmin allele (Fig. 1, *A* and *B*). $des^{+/ml}$ mice were indistinguishable from their wild-type littermates both anatomically and behaviorally. Heterozygotes were crossed to produce mice homozygous for the disrupted allele ($des^{ml/ml}$), and the progeny were identified by Southern analysis (Fig. 1 *B*). Surprisingly, $des^{ml/ml}$ mice were viable and showed the expected Mendelian distribution. Northern and Western blot analysis (Fig. 1, *C* and *D*) on RNA and protein prepared from mouse leg muscle verified the absence of desmin message and protein in mice homozygous for the disrupted allele. $des^{ml/ml}$ mice display no obvious anatomical defects when compared to wild-type and heterozygous littermates, except for a slightly reduced size in a very small percentage of individuals. Both male and female desmin null mice are fertile, although null females produce slightly smaller litter sizes when compared to wild-type females (data not shown).

Analysis of Intermediate Filament and Sarcomeric Protein Expression in Mice Lacking Desmin

To further confirm that mice homozygous for the disrupted desmin allele did not express desmin, we analyzed frozen tissue sections of cardiac, skeletal, and smooth muscle from wild-type and $des^{ml/ml}$ mice by indirect immunofluorescence. Staining of wild-type cardiac muscle with the desmin monoclonal antibody DE-U-10 revealed the expected striated pattern at the Z-disc of the sarcomere, and very prominent expression at the intercalated discs (Fig. 2 *A*, *arrow*). Desmin in wild-type skeletal muscle also demonstrates striated Z-disc staining (Fig. 2 *B*). Sections of cardiac and skeletal muscle from mice homozygous for the disrupted desmin allele did not reveal, as expected, any positive staining for desmin in either tissue (Fig. 2, *F* and *G*). The same results were obtained when tissue sections were stained using a polyclonal anti-desmin antibody (not shown). In wild-type intestinal smooth muscle, desmin can be seen as a filamentous network in cells of the longitudinal and circular layers of muscle, as well as in individual smooth muscle cells extending up into the villi of the intestine (Fig. 2 *C*, *arrow*). Desmin staining is completely absent in smooth muscle from $des^{ml/ml}$ mice (Fig. 2 *H*).

During embryonic development of skeletal muscle, levels of desmin expression increase as terminal differentiation proceeds, and desmin filaments reorganize at the periphery of the Z-discs (Granger and Lazarides, 1978; Gard and Lazarides, 1980). Vimentin expression is initially high in replicating myoblasts, but gradually decreases as terminal differentiation occurs (Bennett et al., 1979; Gard and Lazarides, 1980). Like desmin, vimentin can be detected at the Z-discs at low levels, but it gradually disappears and is at very low levels or totally absent from adult skeletal muscle (Tokuyasu et al., 1984). Another IF, nestin, has also been shown to be present in replicating myoblasts and early myotubes, but like vimentin, it is also absent in adult skeletal muscle (Sejernsen and Lendahl, 1993; Kachinsky et al., 1994). Recent knockouts of keratins 14 and 10 have suggested that in the absence of these IF subunits, expression of other keratin IFs are able to at least partially compensate for the loss of these subunits (Lloyd et al., 1995; Porter et al., 1996). To determine if nestin and vimentin are expressed in skeletal muscle of adult desmin null mice, we stained sections of tongue (Fig. 2, *D*, *E*, *I*, and *J*) and thigh (not shown) muscle with a monoclonal nestin antibody and a polyclonal vimentin antibody. Nestin expression is completely absent in adult desmin null skeletal muscle as it is in wild-type muscle (Fig. 2, *E* and *J*). Likewise, vimentin staining is absent in null myofibers as it is in myofibers from wild-type muscle. Vimentin staining can be seen in mononucleated fibroblasts and presumptive sat-

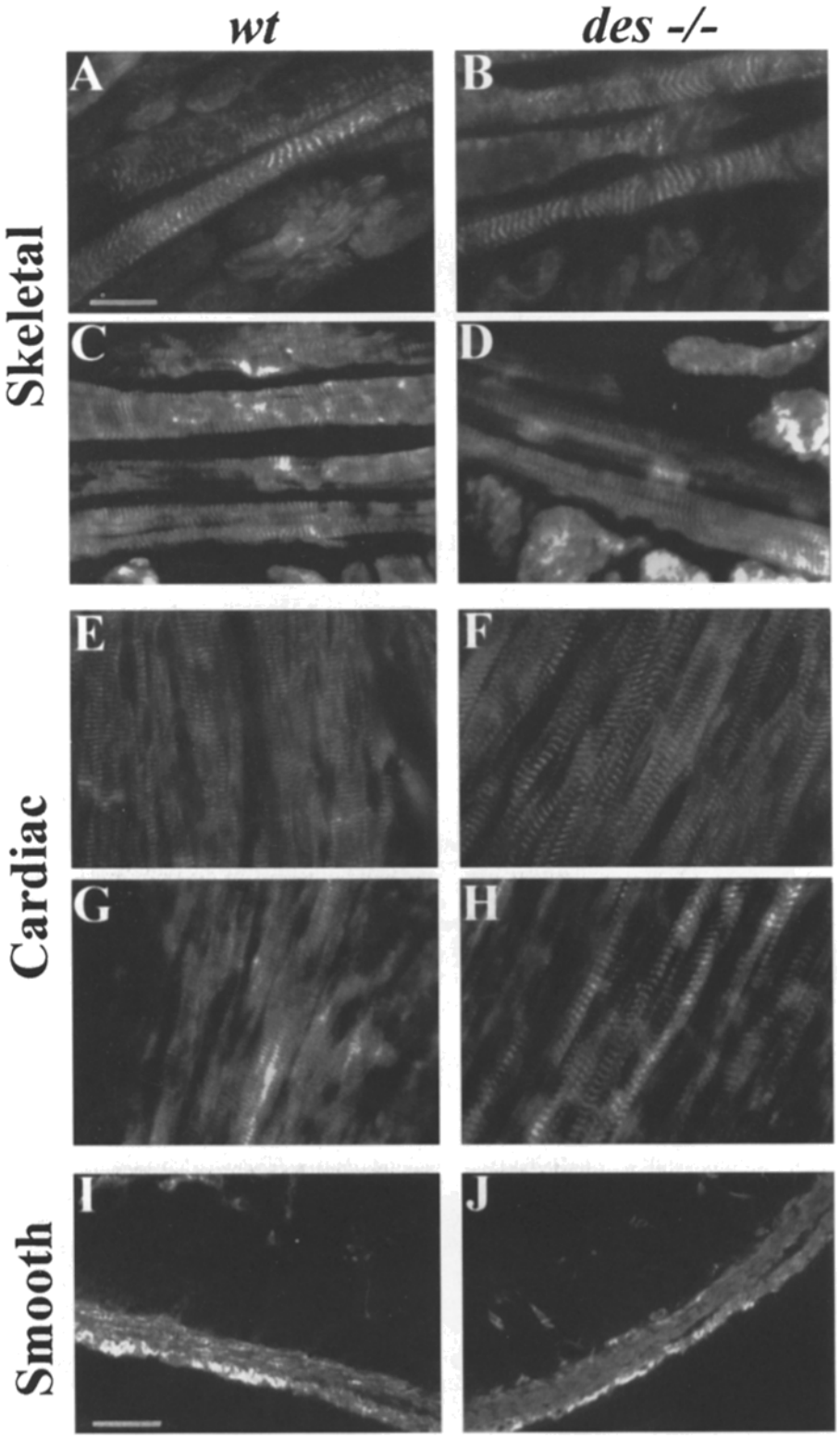


Figure 3. Analysis of sarcomeric protein expression by immunofluorescence. Frozen sections from the tongue (*A–D*), heart (*E–H*), and gut (*I* and *J*) were stained for α-actin (*A*, *B*, *E*, and *F*), tropomyosin (*C*, *D*, *G*, and *H*), and smooth muscle α-actin (*I* and *J*). Sections from desmin null mice were taken from regions showing little or no degeneration in order to give an accurate comparison with wild-type muscle. No detectable difference in localization or intensity of staining was observed for any of the antigens investigated between wild-type (*A*, *C*, *E*, *G*, and *I*) and desmin null (*B*, *D*, *F*, *H*, and *J*) muscle. Bars: (*A–H*) 25 μm; (*I* and *J*) 40 μm.

ellite cells (Fig. 2, *D* and *I*; *arrows*) in both wild-type and null skeletal muscle. The number of vimentin positive mononucleated cells is somewhat higher in null skeletal muscle when compared to wild-type muscle, especially in the thigh (not shown), possibly due to proliferation of satellite cells in damaged areas (see below). Northern (Fig. 1 *C*, *bottom panel*) and Western (Fig. 1 *D*, *bottom panel*) analysis of vimentin expression from adult thigh muscle also showed no detectable increase of vimentin expression in desmin null muscle.

We also stained wild-type and desmin null cardiac muscle for the presence of vimentin. As in the case of skeletal muscle, no vimentin expression could be detected in cardiac muscle of either wild-type or desmin null mice. Recently, nestin has been shown to be very transiently expressed in midembryonic cardiac myofibers (Kachinsky

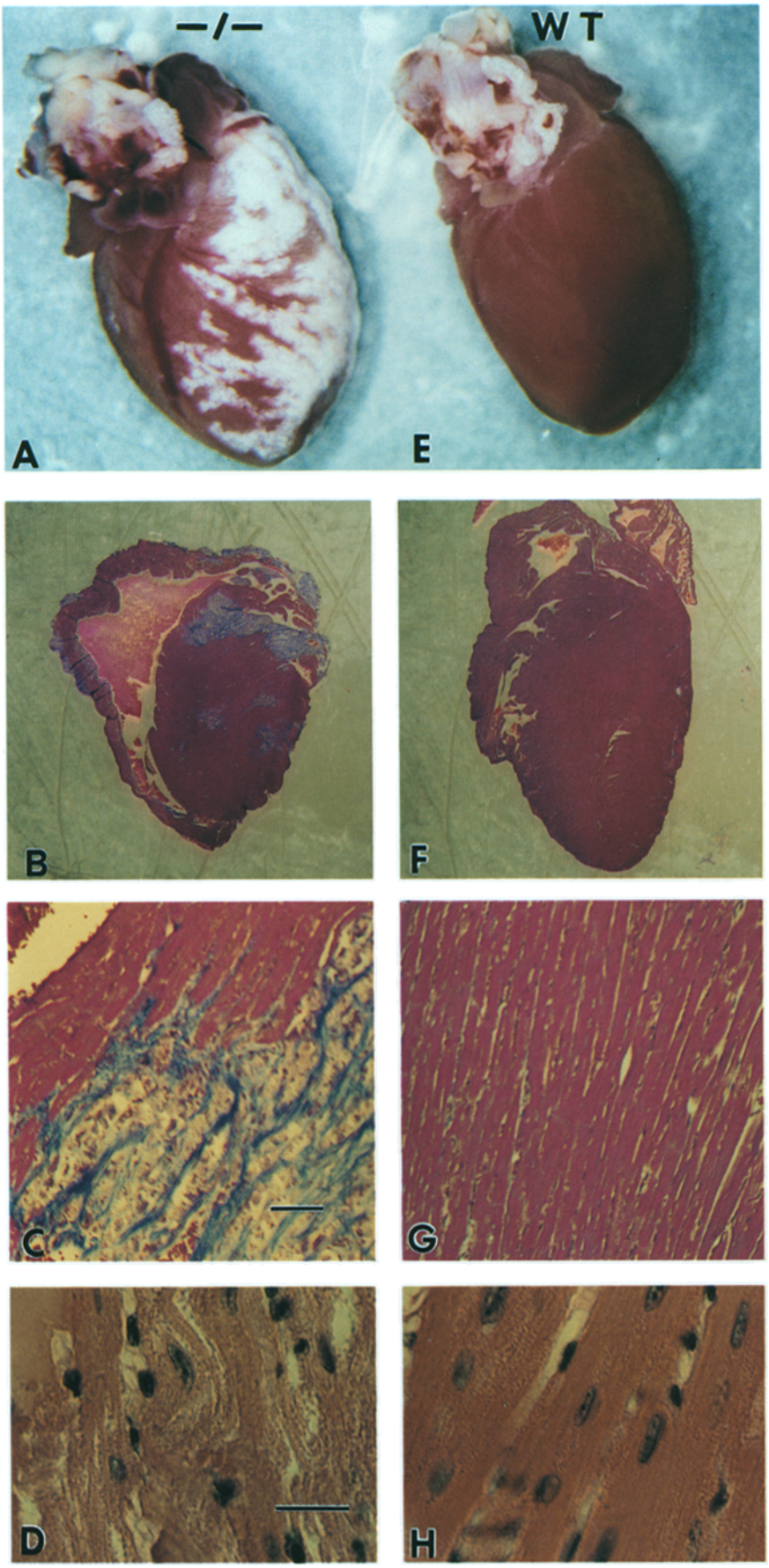


Figure 4. Whole hearts and cardiac muscle sections from mice lacking desmin. Hearts from 10-wk-old desmin null (*A*) and wild-type (*E*) mice demonstrate extensive areas of degeneration and calcification (as confirmed by VonKossa staining in Fig. 5) throughout the myocardium of $des^{-/-}$ mice. Sections from desmin null (*B* and *C*) and corresponding areas in wild-type mice (*F* and *G*) were stained with Masson's trichrome to reveal areas of interstitial fibrosis and degeneration. High magnification of cardiac myofibers taken from nonfibrotic regions of desmin null myocardium (*D*) and wild-type (*H*) myocardium stained with hematoxylin and eosin. Several of the fibers from the desmin null myocardium appear to be disrupted and disintegrating. Bars: (*D* and *H*) 5 μm; (*C* and *G*) 10 μm.

et al., 1995). Staining of cardiac muscle from both wild-type and null mice for nestin also showed no staining (data not shown). Thus, we conclude that in the absence of desmin, neither vimentin nor nestin expression is maintained in adult striated muscle.

As myofibrils are assembled in *des*$^{m1/m1}$ mice, it is obvious that the presence of desmin is not necessary for the assembly of these structures. However, the absence of desmin might influence the localization or level of expression of individual myofibrillar proteins. To address this question, we stained tissue sections of muscle from wild-type and desmin null mice for a number of proteins found in the contractile apparatus. To make an accurate comparison, we only analyzed regions of muscle from desmin null mice which had not yet undergone any degeneration (see below). In striated muscle, sarcomeric α-actinin is a major component of the Z-disc. We were unable to detect any discernible difference in localization or intensity of α-actinin staining in wild-type or desmin null cardiac or skeletal muscle (Fig. 3, *A*, *B*, *E*, and *F*). Similar results were obtained from staining of cardiac and skeletal muscle for tropomyosin (Fig. 3, *C*, *D*, *G*, and *H*). Tropomyosin is not localized along the Z-lines itself but appears on either side of the Z-line, thus displaying a wider band. No obvious differences between wild-type and *des*$^{m1/m1}$ can be seen, indicating that the absence of desmin does not influence the localization or expression of this protein. Similar results were obtained when sections were stained for nebulin (not shown) and myosin heavy chain (not shown). Staining of intestinal smooth muscle tissue sections for the smooth muscle isoform of α-actin also showed no obvious difference in staining between desmin null and wild-type mice (Fig. 3, *I* and *J*). Thus, we conclude that the absence of desmin does not influence the localization and levels of expression of these proteins of the contractile apparatus.

Multiple Muscle Disorders Accompany Disruption of Muscle Architecture in Mice Lacking Desmin

Further analysis of *des*$^{m1/m1}$ mice revealed defects in all three muscle types. While most *des*$^{m1/m1}$ mice are anatomically identical to wild-type and heterozygous mice, by the second week after birth we noted that *des*$^{m1/m1}$ mice were somewhat more lethargic than their wild-type and heterozygous littermates. Dissection of *des*$^{m1/m1}$ mice revealed the presence of extensive calcium deposits in the myocardium, with higher concentrations on the left ventricle (Fig. 4 *A*). Confirmation that the deposits contained calcium

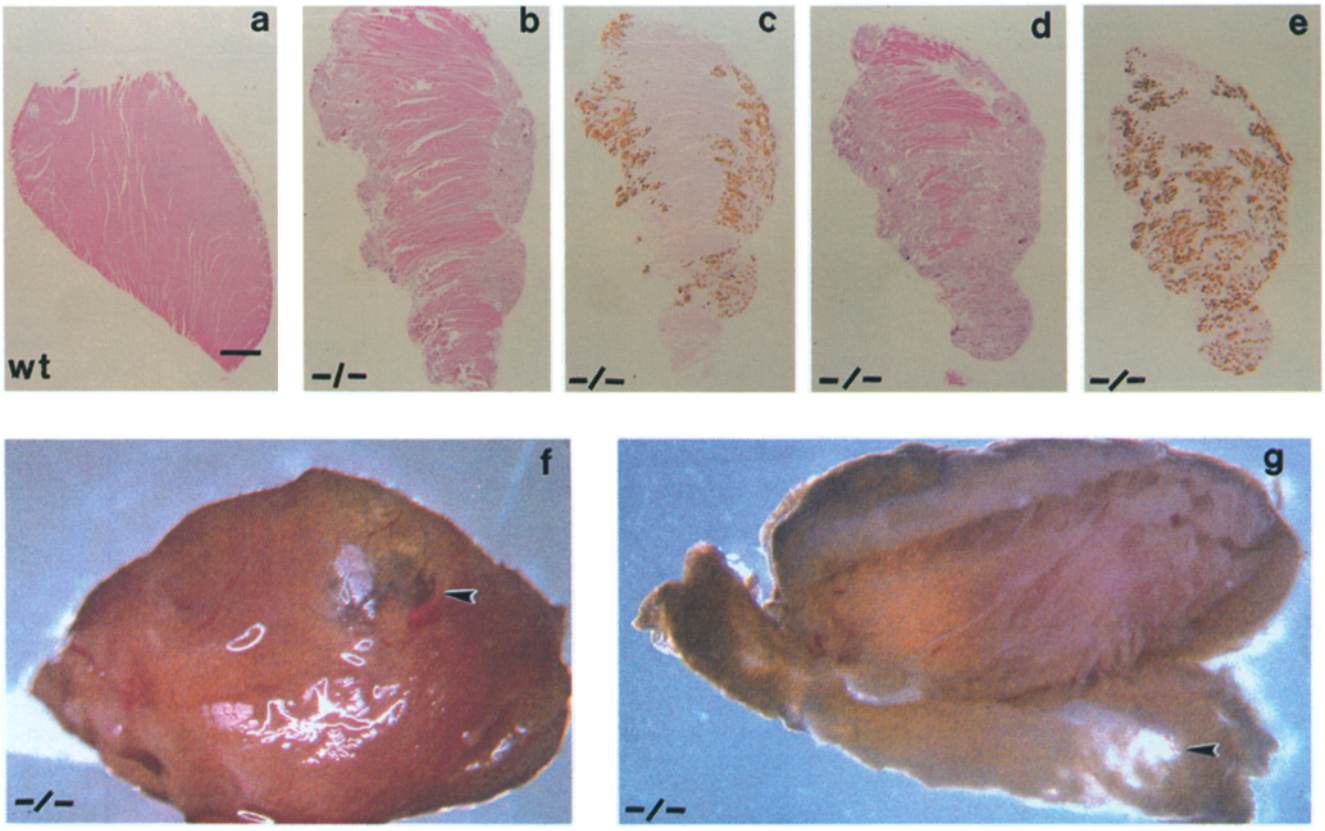


Figure 5. Progressive degeneration of cardiac muscle in mice lacking desmin. Tissue sections from 10-wk-old wild-type (*a*) and desmin null (*b–e*) myocardium stained with hematoxylin and eosin (*a*, *b*, and *d*) or VonKossa's stain (*c* and *e*). Wild-type muscle shows no degeneration, but desmin null myocardium shows extensive degeneration and fibrosis. The extent of degeneration is more severe in superficial sections of the myocardium (*d* and *e*) than in sections taken deeper within the myocardium (*b* and *c*). The brown precipitate formed in sections stained with VonKossa's (*c* and *e*) stain indicates the presence of calcium deposits. The degeneration of cardiac tissue increases with age. A heart removed from an 11-wk desmin null mouse (*f* and *g*) shows extensive degeneration. Note the absence of calcium deposits in this heart. The degeneration is so severe that some areas of the myocardium appear transparent (*arrowhead*, *f* and *g*). (*g*) The same heart with the external scar tissue of the myocardium cut and pulled back to reveal damaged cardiac muscle. Bar: (*a–e*) 10 μm.

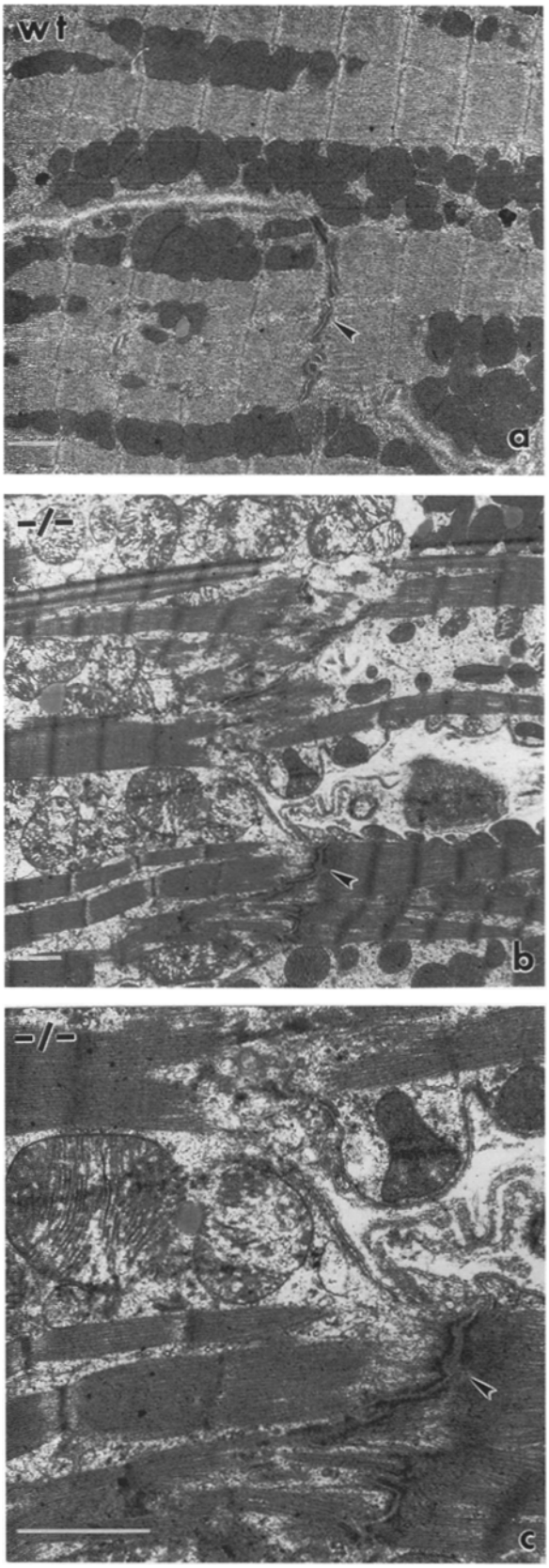


Figure 6. Transmission electron microscopy of cardiac muscle from mice lacking desmin. Wild-type cardiac muscle (*a*) shows aligned arrays of myofibrils terminating and inserting at intercalated discs (*arrowhead*). Myofibrils are interrupted occasionally by packed strands of mitochondria. A number of fibers from desmin null myocardium (*b*) show disorganization and signs of damage. The myofibrils are disorganized and in some cases appear to have separated from intercalated discs (*arrowhead*). Note that the mitochondria are also highly disorganized, and many appear to be swollen. (*c*) Higher magnification view of *b*. Bars, 1.0 μm.

was provided by VonKossa staining of cardiac tissue sections (Fig. 5, *c* and *e*). Histological (Fig. 4, *B*, *C*, *D*, *F*, *G*, and *H*; 5, *a–e*) and electron microscopic (Fig. 6) analysis revealed the appearance of a general loss of cardiomyofiber tension, with areas of considerable disruption and disorganization of the cardiac myofibers and myofibrils. Staining with Masson's trichrome for collagen revealed areas of extensive interstitial fibrosis and necrosis (Fig. 4, *B* and *C*). Degeneration of the myocardium was more prominent throughout the left ventricle, and the lesions appeared to progress from the exterior of the myocardium to the interior (Fig. 5, *a–e*), suggesting that over time, the degeneration of cardiac tissue works from the exterior of the myocardium inward (see also Fig. 5, *b–e*).

Occasionally, the extent of muscle loss was very severe and this was easily manifested upon dissection by the transparency of areas of the myocardium, as shown in Fig. 5 (Fig. 5, *f* and *g*). While analysis of cardiac sections from neonates shows little degeneration and no calcification (not shown), these defects are visible by the third week after birth and gradually increase with age. Additionally, in cases of severe lesions the calcium deposits were disappearing together with the degenerating tissue (Fig. 5, *f* and *g*). Electron microscopy of sections from desmin null ventricular muscle revealed that while there are a large number of apparently normal fibers, several myofibers demonstrate severely disrupted architecture (Fig. 6). In contrast to wild-type fibers, which display laterally aligned myofibrils and nicely packed strands of mitochondria (Fig. 6 *a*), these myofibers have lost the normal lateral alignment of myofibrils and often show disrupted myofibrils, particularly at the positions of the intercalated discs (Fig. 6, *B* and *C*) and detachment from the sarcolemma. Additionally, the mitochondria have lost their organization as well, and they often appear to be swollen and disintegrating.

Histological examination of skeletal muscle of the tongue, leg, and diaphragm revealed similar defects in the integrity and organization of the myofibers, although to a lesser extent than that seen in cardiac muscle. Abnormalities included loss of myofiber tension, ragged and disorganized myofibers with few, if any, discernable striations, some loss of stable nuclear positioning and indications of degeneration (Fig. 7). The extent of damage depends on the origin of the muscle examined. In the tongue, a majority of the fibers display the ragged, disintegrating morphology (Fig. 7 *A*), while in muscle from the back, relatively few fibers show this morphology (not shown). Cross sections of skeletal muscle from $des^{ml/ml}$ and wild-type mice (Fig. 7, *C* and *F*) demonstrate that in mice lacking desmin, many fibers are also ragged in appearance, of smaller diameter and frequently not closely packed when compared to wild-type muscle. Additionally, there appears to be more nuclei present in cross sections from null mice, including some fibers which appear to have internal clusters of multiple nuclei. Electron microscopy of skeletal muscle from the diaphragm (Fig. 8) clearly revealed that, as in

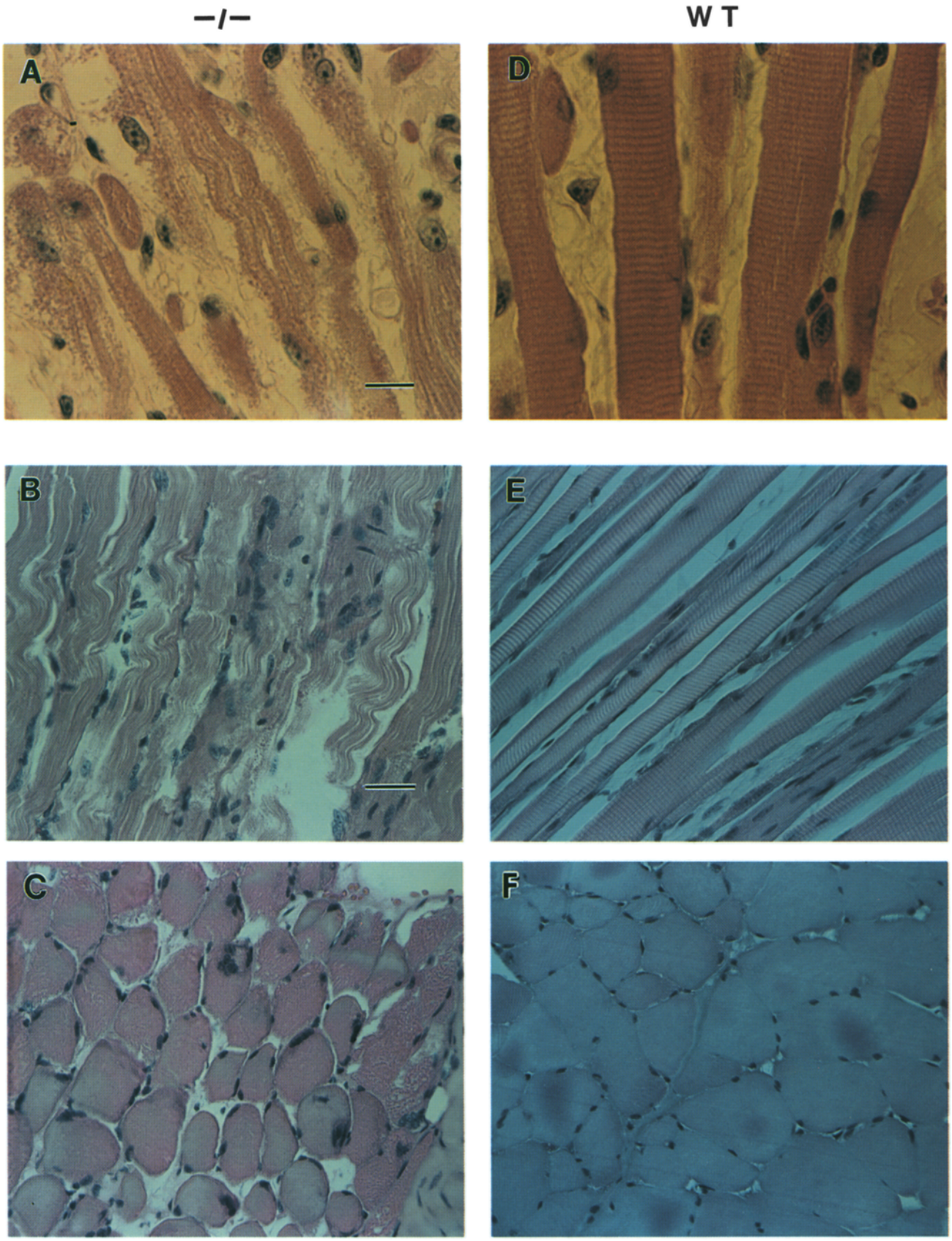


Figure 7. Skeletal muscle abnormalities in desmin null mice. Longitudinal sections of tongue (*A* and *D*) and thigh (*B* and *E*) skeletal muscle from 5-wk-old desmin null (−/−) and wild-type (*WT*) mice. Numerous skeletal muscle fibers in desmin null mice (*A* and *B*) appear ragged and disorganized, giving the impression that they are degenerating when compared to wild-type muscle (*D* and *E*). Striations are rarely discernible in these fibers. Cross sections of thigh skeletal muscle from null (*C*) and wild-type (*F*) mice demonstrate that in des−/− mice, many fibers are also ragged in appearance and are frequently not closely packed when compared to wild-type muscle. Additionally, there appear to be more nuclei present in cross sections from null mice, including some fibers which appear to have multiple nuclei clustering internally. Bars: (*A* and *D*) 5 μm; (*B* and *C*, *E* and *F*) 10 μm.

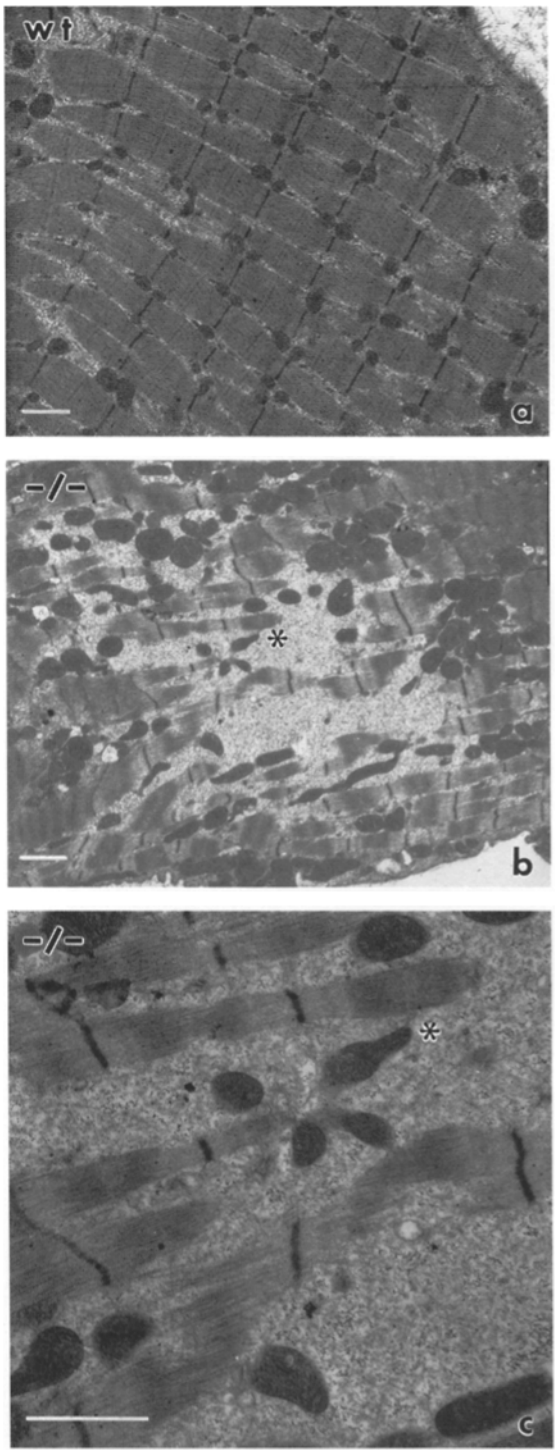


Figure 8. Transmission electron microscopy of skeletal muscle from 10-wk-old desmin null and wild-type mice. Sections of myofibers from wild-type diaphragm (*a*) show well-formed myofibrils in register, and paired mitochondria arranged in an orderly fashion at the Z-lines between adjacent myofibrils. Corresponding sections from desmin null (−/−) diaphragm show numerous areas with severely disrupted myofibrils (*b* and *c*). The star in *b* identifies the area magnified in *c*. The myofibrils are not in register, and are often separated by large areas of cytoplasm. Some myofibrils appear to be disintegrating. The mitochondria are also very disorganized. Bars, 1.0 μm.

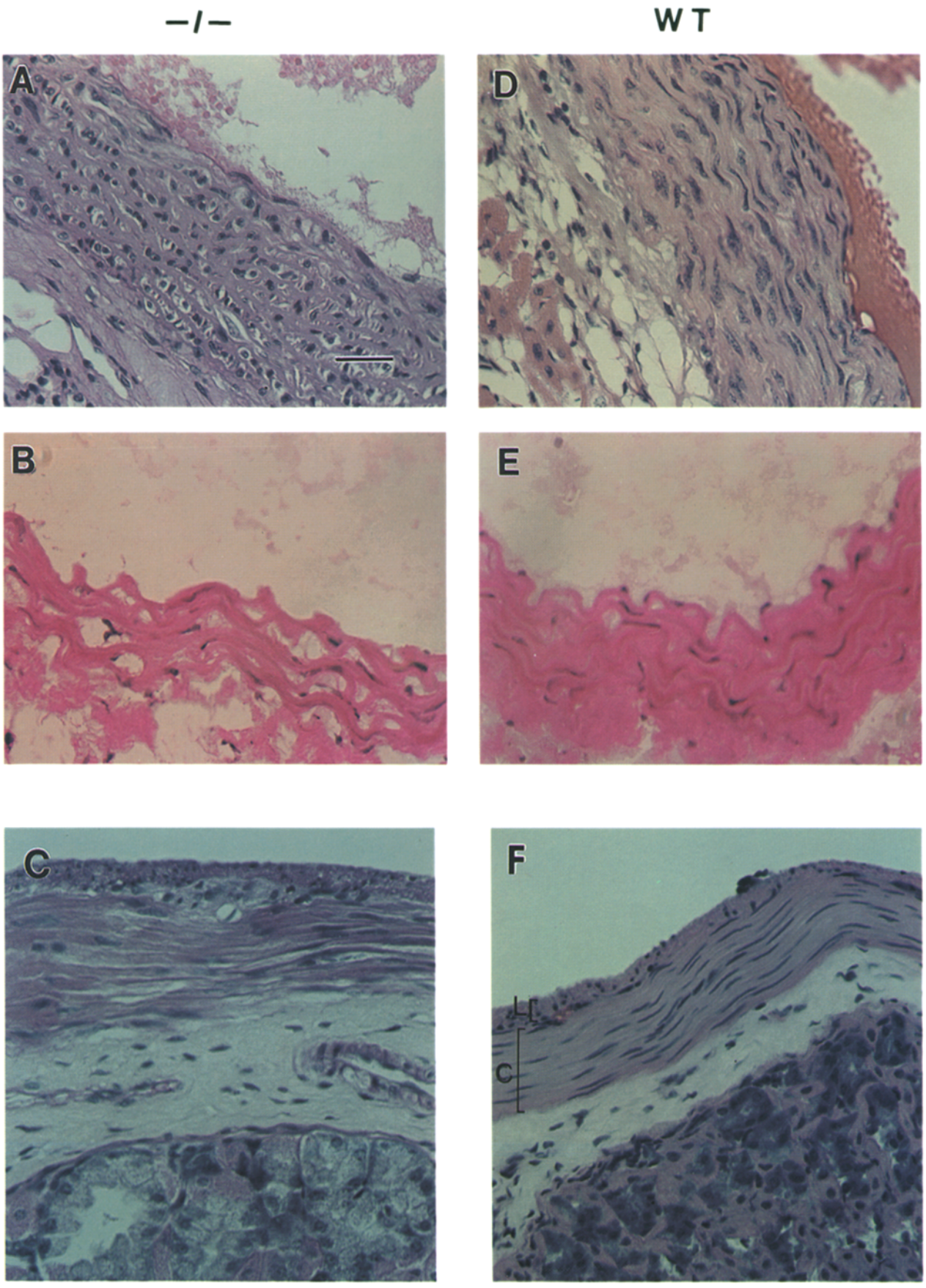
cardiac muscle, some myofibers from $des^{m1/m1}$ animals appear relativley normal, while others show disrupted intracellular architecture, including abnormally registered sarcomeres with missing or disoriented Z-discs and an absence of typically striated A and I bands. Similarly, an additional severe effect was the disorganization of mitochondria. While the mitochondria are found in pairs at the Z-line in wild-type diaphragm, this organization is lost in a number of fibers in $des^{m1/m1}$ muscle. Additionally, a number of mitochondria also show the swollen, disintegrating morphology similar to those seen in some $des^{m1/m1}$ cardiac myofibers (Fig. 6, *b* and *c*). Indeed, association of mitochondria with intermediate filament networks has been reported in previous investigations (Tokuyasu et al., 1983*a*,*b*). Among the different skeletal muscle types studied, tongue demonstrated the most severe myofiber disruption and disintegration both by histological (Fig. 7 *A*) and electron microscopic analysis (data not shown). The diaphragm was also severely affected, while skeletal muscle from the thigh is affected to a somewhat lesser degree. The muscles of the back show the least disruption and degeneration (not shown). These results suggest a possible correlation between the severity of the damage and the extent of muscle usage.

Histological analysis also revealed parallel defects in smooth muscle. As shown in Fig. 9, abnormalities of the smooth muscle of the aorta from $des^{m1/m1}$ mice include loose organization of smooth muscle cells, a preponderance of empty space around the myofiber nuclei, hypoplasia, and a low degree of degeneration at both the outtrack (Fig. 9 *A*) and the thoracic levels (Fig. 9 *B*). The clearing of cytoplasm around the nuclei can also be observed at the electron microscopic level (not shown). The effect of the absence of desmin was also examined in gastric smooth muscle. The defects observed here were generally milder than those seen in other muscles. We observed partial loss of the outer longitudial smooth muscle layer and some hypoplasia of the circular layer muscle of the intestine. Loose cellular organization and adhesion was also very pronounced (Fig. 9 *C*).

Discussion

Function of Desmin in Muscle Maintenance and Strength vs Muscle Formation

The present data demonstrate that in the absence of desmin prenatal muscle development can take place in mice, but muscle integrity and function cannot be maintained. The former data were surprising for two reasons. First desmin is expressed very early during both cardiac and skeletal muscle development preceding the expression of most myogenic HLH regulators (Schaart et al., 1989), thus suggesting an important role in muscle formation. Second, in vitro studies using both antisense RNA techniques (Li et al., 1994), as well as desmin null embryoid bodies differentiating in vitro (Weitzer et al., 1995), demonstrated the importance of desmin at least in skeletal and smooth muscle development. Obviously, the present in vivo data do not support the previously reported in vitro data. However, the existing difference can be explained by the potential use of vimentin or nestin in vivo to compensate for the absence of desmin during development. Such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compensatory mechanisms seem to be very efficient in vivo not only for members of the IF family (Lloyd et al., 1995; Colucci-Guyon et al., 1994; Gomi et al., 1995; Porter et al., 1996) but also for several other genes, particularly in muscle (Olson and Klein, 1994). In embryoid bodies, however, these compensatory mechanisms seem to take place effectively only in cardiac and not in skeletal and smooth muscle development (Weitzer et al., 1995). In addition to vimentin and desmin, nestin is also expressed in both skeletal and cardiac early muscle development (Kachinsky et al., 1994, 1995). Generation of a double null mutation of desmin and vimentin and/or nestin will directly address whether or not either of these two IF proteins can compensate for the absence of desmin during development in vivo. However, an additional complication is the recognition that there is now a fourth IF protein, synemin, expressed in muscle tissue (Granger and Lazarides, 1980; Becker et al., 1995). Like desmin, synemin also shows similar localization at the Z-line in striated muscle (Granger and Lazarides, 1980; Muguruma et al., 1981; Price and Lazarides, 1983). Unfortunately, addressing the issue of whether synemin can compensate for the absence of desmin during myogenesis in vivo or partially compensate for its absence in adult muscle is impossible at this time, as mouse synemin has not yet been cloned and antibodies recognizing mouse synemin are not yet available.

It was clearly demonstrated that *des$^{ml/ml}$* mice have defects in all three muscle types. Common features in all cases include overt cell and tissue damage with signs of instability and degeneration. The effects of the absence of desmin are most severe in cardiac muscle, with smooth muscle seemingly least affected and skeletal muscle exhibiting damage that is intermediate in severity. The defects we see are most possibly due to either increased fragility of the myofibers, or to impairment of normal myofibril and myofiber repair and regeneration (Grounds and Yablonka-Reuveni, 1993). Additionally, these results seem to suggest that the more active the muscle tissue, the greater damage and degeneration it exhibits in the absence of desmin. This is supported by the fact that degeneration of cardiac muscle, which is constantly contracting, is the most severe that we observe, and that there seems to be a range of severity of degeneration seen in skeletal muscle. Very active muscles such as the diaphragm and tongue show a higher amount of degeneration than muscles which are less active, such as those of the back. However, only a fraction of the cell population in a given muscle tissue is defective. This indicates that desmin is not absolutely necessary for the formation and assembly of myofibrils, but rather it suggests that it is required for maintenance of their integrity and possibly for their repair or regeneration after mechanical damage. These data are consistent with the hypothesis that IFs form a transcytoplasmic integrating matrix that might contribute to the integrity and strength of both myofibrils and myofibers (Lazarides, 1980; Lazarides and Capetanaki, 1986). Disruption of IFs in cultured muscle cells using truncated desmin dominant mutants in transient transfection experiments (Schultheiss et al., 1991) agree with the present data in which desmin is not absolutely necessary for myofibril assembly and initial lateral alignment, as was also suggested originally by Hill and colleagues (Hill et al., 1986) Those studies, however, could not address issues regarding the role of IFs in the maintenance and proper integrity of these structures in the tissue. On the other hand, disruption of IFs by microinjection of DNA coding for truncated vimentin or desmin in fertilized *Xenopus* eggs also did not seem to influence myofibril assembly but destabilized the intersomite junction in myotomal muscle (Cary and Klymkowsky, 1995).The role of IFs in cell integrity and strength has been suggested from numerous studies with keratins (Coulombe et al., 1991*a*,*b*; Bonifas et al., 1991; Vassar et al., 1991; Cheng et al., 1992; Chipev et al., 1992; Lane et al., 1992; Rothnagel et al., 1992; Reis et al., 1994; Torchard, 1994; for review see Fuchs, 1994; McLean and Lane, 1995). Furthermore, transgenic mouse models have provided evidence directly linking neurofilament abnormalities to neurodegenerative processes (Cote et al., 1993; Xu et al., 1993; Lee et al., 1994; Collard et al., 1995; for review see Lee and Cleveland, 1994.). Thus, muscle that is deficient in desmin could be more fragile than wild-type muscle, and as such, would be more easily damaged during contractile activity. This would lead one to expect that more actively contracting muscle would incur more damage and thus undergo more extensive degeneration.

An alternative explanation for the severity of the degeneration seen in cardiac muscle is that a vascular abnormality resulting from the absence of desmin in smooth muscle results in reduced blood supply to the myocardium, leading to progressive degeneration. This possibility is supported by the observed effect that the absence of desmin has on the smooth muscle of the aorta. On the other hand, the possibility that the primary cause of myocardial degeneration is fragility of the myofibers, is supported by the observed effects on skeletal muscle. However, these possibilities are not mutually exclusive, and could both contribute to the observed myocardial degeneration. Overall, the phenotype observed in smooth muscle was less dramatic (except in the case of the aorta) than that seen in cardiac and skeletal muscle. As with striated muscle, the abnormalities observed in smooth muscle could be due to tissue instability as a consequence of the absence of desmin and/or an inability to repair mechanical damage resulting from normal activity of the muscle. The fact that organs containing smooth muscle are less affected may be due to the sustained expression of vimentin which, in contrast to striated muscle, is maintained after differentiation (Schmid et al., 1982). Very preliminary examination of uterine smooth muscle did not demonstrate extreme differences between nonpregnant wild-type and des$^{ml/ml}$ tissue. However, uteri

Figure 9. Defects in smooth muscle from mice lacking desmin. Sections of aorta from the outtrack (*A* and *D*) and thoracic (*B* and *E*) levels of desmin null (−/−) and wild-type (*WT*) mice. Note the clearing of cytoplasm around myofiber nuclei (*A*) and hypoplasia of the smooth muscle layer (*B*) in aortic sections from desmin null mice. Some areas between elastic fibers in the null aorta (*B*) appear devoid of cells. A loose organization of tissue can be seen in the circular (*C*) muscle layer of the stomach from desmin null mice, as well as a slightly reduced thickness of the outer longitudinal (*L*) layer of muscle of the stomach when compared to wild-type tissue (*F*). Bar, 10 μm.

from pregnant $des^{m1/m1}$ females show marked hypoplasia of smooth muscle compared to pregnant wild-type uterine tissue (data not shown).

As in dystrophin deficiencies that lead to muscle degeneration (Jackson, 1993), the causes of the observed muscle degeneration in the absence of desmin are not clear at this point. They could be the consequence of tissue damage due to muscle weakness in the absence of desmin, and/or an inability of the $des^{m1/m1}$ mice to efficiently repair damage caused by normal mechanical stress. Such defects in damage repair can be correlated with delayed wound healing found in BPAG1, or keratin bundling protein, knockout mice (Guo et al., 1995), possibly due to abnormal keratin filament behavior. The latter case could be due to defects in signaling which might be important for both myofibril and myofiber repair and regeneration. This possibility is supported by recent reports from GFAP null mice, which have suggested involvement of the glial cell specific IF in communication between Bergmann glia and Purkinje cells (Shibuki et al., 1996).

Desmin and Muscle Disease

There are several reported myopathies and cardiomyopathies with abnormally accumulated granular and filamentous aggregates of desmin (for review see Goebel and Borneman, 1993). There is no case, however, where desmin is completely absent. Similarly, there is no evidence, so far, demonstrating that these abnormalities in desmin distribution have a causal effect on the disease. Muscle damage and degeneration is the hallmark of most common myopathies, cardiomyopathies, and muscular dystrophies (Grounds and Yablonka-Reuveni, 1993). In addition, several of the lesions developed in all three muscle types of the desmin null mice are reminiscent of those reported in some familial desminopathies (Vajsar et al., 1992; Horowitz and Schmalbruch, 1993; Ariza et al., 1995; for review see Goebel and Borneman, 1993). These include dilation of the ventricle chamber (Figs. 4 and 5), hypoplasia of the aorta, thinned intestinal walls, and distended and fragmented Z-lines (Ariza et al., 1995). The present data support the idea that the observed abnormalities in desmin distribution in several reported myopathies might have a causal effect. Such hypothesis is favored by the extensive similarity of the desmin abnormal aggregates to those of keratin filament aggregates which relate directly to different skin diseases (Coulombe et al., 1991*a*,*b*; Bonifas et al., 1991; Vassar et al., 1991; Cheng et al., 1992; Chipev et al., 1992; Lane et al., 1992; Rothnagel et al., 1992; Reis et al., 1994; Torchard et al., 1994; for review see Fuchs, 1994; McLean and Lane, 1995). Furthermore, natural human mutations in k14 (Chan et al., 1994; Rugg et al., 1994) and the knockout of the k14 gene (Lloyd et al., 1995) provided further support for the notion that cell fragility is a consequence of the absence of the keratin filament network rather than the presence of insolable aggregates. Consequently, it could be suggested that the desmin null mouse can provide an animal model for several skeletal and cardiac myopathies which closely resemble those with abnormalities in desmin distribution (Thornell et al., 1980, 1983; Goebel and Bornemann, 1993; Vajsar, 1993; Horowitz and Schmalbruch, 1994; Ariza et al., 1995).

We thank Eva Regel for assistance with blastocyst microinjections and Liz Hopkins for assistance with histology. We thank Dr. William Brinkley, Dr. Michael Mancini, and Donna Turner in the Integrated Microscopy Core Laboratory for enormous help with electron microscopy and imaging. We thank Dr. Maureen Price, Dr. Stavros Topouzis, Dr. Mark Entman, Dr. Howard Holtzer, Dr. Henry Epstein, Dr. Michael Schneider, and Dr. Leslie Leinwand for valuable discussions. A short communication on the desmin knockout was reported during the final preparation of this manuscript by Li et al. (1996).

This work was supported by National Institutes of Health grant AR 39617-01 and a Muscular Dystrophy Association grant to Y. Capetanaki and a Max Kade award to G. Weitzer.

Received for publication 5 June 1996 and in revised form 2 July 1996.

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