Dilated cardiomyopathy (DCM) is characterised by dilatation of cardiac chambers and impaired contraction, leading to heart failure or sudden death. Symptoms are highly variable in severity and age of onset. Penetrance has been found to be incomplete and age related in most families. 1–3 DCM appears to be a complex genetic disorder, with 15 genes being implicated in autosomal dominant DCM (OMIM #115200). What is remarkable about DCM is that a variation in one of these genes does not result exclusively in a DCM phenotype. In particular, lamin A/C (LMNA) mutations have also been described in at least nine other diseases, including Emery-Dreifuss and other lamin A/C (LMNA) gene variations have been reported in more than one third of genotyped families with dilated cardiomyopathy (DCM). However, the relationship between LMNA mutation and the development of DCM is poorly understood.

Methods and results: We found that end stage DCM patients carrying LMNA mutations displayed either dramatic ultrastructural changes of the cardiomyocyte nucleus (D192G) or nonspecific changes (R541S). Overexpression of the D192G lamin C dramatically increased the size of intranuclear speckles and reduced their number. This phenotype was only partially reversed by coexpression of the D192G and wild type lamin C. Moreover, the D192G mutation precludes insertion of lamin C into the nuclear envelope when co-transfected with the D192G lamin A. By contrast, the R541S phenotype was entirely reversed by coexpression of the R541S and wild type lamin C. As lamin speckle size is known to be correlated with regulation of transcription, we assessed the SUMO1 distribution pattern in the presence of mutated lamin C and showed that D192G lamin C expression totally disrupts the SUMO1 pattern.

Conclusion: Our in vivo and in vitro results question the relationship of causality between LMNA mutations and the development of heart failure in some DCM patients and therefore, the reliability of genetic counselling. However, LMNA mutations producing speckles result not only in nuclear envelope structural damage, but may also lead to the dysregulation of cellular functions controlled by sumoylation, such as transcription, chromosome organisation, and nuclear trafficking.

METHODS

Subjects, heart tissues, and LMNA screening
Written informed consent was obtained from 92 DCM probands and their relatives in accordance with study protocols approved by hospital ethics committees.

Each amplified DNA fragment from LMNA was submitted to both single strand conformational polymorphism electrophoresis and denaturing high performance liquid chromatography (DHPLC) (Helix, Variant) analysis.

Detailed descriptions of patient and family recruitment, heart tissue collection and processing, and screening methods are provided as supplementary online information at http://jmg.bmj.com/content/supplemental.

Construction of expression plasmids
Full length lamin C cDNA was obtained by PCR amplification of first strand cDNAs (forward primer 5'-GGAATTCATGGAGACCCCGTCACCAGCG-3', reverse primer 5'-GCGGATCCTCA-GCGGGCTACCA-3'). The amplicon was inserted in the multiple cloning sites of both pECFP-C1 (cyan) and pEGFP-N1 (green) plasmids.

Abbreviations: DCM, dilated cardiomyopathy; SUMO1, small ubiquitin-like modifier 1
pEYFP-C1 (yellow) vectors (Clontech Laboratories). The cDNA encoding wild type prelamin A, cloned into pcDNA4 (Invitrogen) was kindly provided by Drs Morris and Holt (North East Wales Institute, UK). The vector was transformed in XL competent Escherichia coli. Point mutations found in DCM patients were introduced by using the Transformer site directed mutagenesis kit (Clonetech Laboratories). SUMO1 amplified by PCR was fused to pEYFP-C3. Deletion of the five C-terminal residues in the pEYFP:SUMO1 construct was achieved by replacing glycine 97 with a stop codon. All clones were systematically sequenced.

Cell culture and transfection
COS7 cells (ATCC) were cultured on glass coverslips in Dulbecco’s modified Eagle’s medium glutamax, supplemented with 10% fetal bovine serum and 1% l-glutamine. The transfection of fusion protein constructs was made using 0.5 μg of expression vectors and Lipofectamine 2000 (Life Technologies). Cells were grown for 17–21 hours. Both cyclohexamide (28 μg/ml) and Hoechst 33258 Dye (10 μg/ml) were added for 30 minutes prior to microscopic observation to halt transcription and visualise chromosomes, respectively. Excitation wavelengths were 434 nm for CFP:lamin C, 514 nm for YFP:lamin C, YFP:SUMO1, and 345 nm for YFP.

Figure 1 Pedigree of the families with LMNA mutations and location of the identified mutation on a schema of LMNA primary sequence. Arrow indicates proband; black filled symbol, dilated cardiomyopathy; open symbol, asymptomatic; grey filled symbol, no clinical data; left half filled symbol (black), left ventricular dilatation; right half filled symbol (black), right bundle branch block; left half filled symbol (grey), heart failure; right half filled symbol (grey), sudden death; black filled dot within symbol, unknown dilated cardiomyopathy status; grey filled dot within symbol, muscular dystrophy; diagonal lines, death; + or – signs, presence or absence of mutations.
Functional significances of novel lamin gene mutations in heart failure patients

Four mutations in four unrelated DCM families were found. In total, 92 DCM probands were screened for variations within LMNA coding sequence and exon–intron boundaries. Four mutations in four unrelated DCM families were found. All mutations were located in highly conserved regions of the gene shared by lamin A and C isoforms. Expressed phenotypes in the 22 mutation carriers were highly variable between families and within families. There were 10 symptomatic mutation carriers, while four adult mutation carriers (age range 23–43 years) and four children (age range 6–14 years) were asymptomatic (clinical characteristics of mutation carriers are provided as supplementary online information at http://jmg.bmjjournals.com/supplemental/).

RESULTS
LMNA mutations and clinical characteristics of mutations carriers
In total, 92 DCM probands were screened for variations within LMNA coding sequence and exon–intron boundaries. Four mutations in four unrelated DCM families were found. All mutations were located in highly conserved regions of the gene shared by lamin A and C isoforms. Expressed phenotypes in the 22 mutation carriers were highly variable between families and within families. There were 10 symptomatic mutation carriers, while four adult mutation carriers (age range 23–43 years) and four children (age range 6–14 years) were asymptomatic (clinical characteristics of LMNA mutation carriers are provided as supplementary online information at http://jmg.bmjjournals.com/supplemental/). Symptomatic mutation carriers were characterised by poor prognosis. In total, of 22 symptomatic family members, 5 patients received heart transplant and 12 died from DCM.

The R190W mutation (C780T transition) was found in a large family from the UK with autosomal dominant inheritance (fig 1). Clinical features for some of the family members have been detailed in a previous publication.16 The D192G mutation (A787G transition) (GenBank accession number AY847595) was found in a Polish family with suspected autosomal dominant transmission (fig 1). The Y481stop mutation (C1656G transversion) (GenBank accession no. AY847596) was found in an unrelated Polish patient (fig 1) and the R541S mutation (C1833A transversion) (GenBank accession no. AY847597) in a Canadian family with suspected autosomal dominant transmission (fig 1).

Pathological studies of hearts from patients with end stage DCM
Light microscopy of the two available heart samples from patients carrying D192G and R541S LMNA mutations revealed non-specific myocyte damage and interstitial fibrosis (data not shown). Electron micrographs of the heart tissue sections from the D192G LMNA patient demonstrated dramatic morphological alterations, including a complete loss of the nuclear envelope (fig 2A), the accumulation of mitochondria, glycogen and/or lipofuscin in the nucleoplasm, and chromatin disorganisation (fig 2B) in approximately 30% of nuclei. By contrast, examination of the heart tissue sections from the patient with R541S LMNA revealed only non-specific nuclear membrane alterations (fig 2C), comparable with those found in transplanted DCM patients without LMNA mutation (fig 2D). Immunostaining with mouse antihuman lamin A + C monoclonal antibody showed normal staining of interstitial, vascular, and myocyte nuclei for both the D192G and R541S patient hearts (fig 2E,F,G).

Effects of overexpression of mutated LMNA in COS7 cell model
To characterise the consequences of LMNA mutations at the cellular level, transient cell transfections were performed on COS7 cells with wild type and/or mutated lamin C mRNA expressed as fusions to the C-terminus of variants of the Aequorea victoria green fluorescent protein (GFP) (lamin C-FP). Wild type and mutated lamin C-FP appeared to strictly colocalise with Hoechst 33258 chromosome dye in the nucleus (fig 3A). Wild type lamin C-FP formed distinct intranucleoplasmic foci in most cells. This individualisation of the intranucleolar granules in numerous discrete speckles was previously observed in Swiss 3T3 and SW13 cells.11–12 (fig 3A). Timelapse video fluorescence microscopy indicated that the intranuclear structures were relatively stable (up to 5 hours) and showed no obvious alteration in number, shape, or location over the time period. In a few cells, we observed a smaller number of spots, which were much larger and tended to be round. During transient expression of D192G lamin C-FP, the number of cells displaying a few large fluorescent rounded spots was dramatically increased compared with wild type lamin C-FP (p<0.0001; fig 3A,C). Indeed, a single giant round spot was present in the nucleus of a mean (SD) 84 (6)% of cells. This spot appeared to be stable in shape and...
location within the nucleus, as indicated by overnight
timelapse videos. Transfection efficiency range was 30–60%.
Western blotting quantification of endogenous versus trans-
fected lamin showed that overexpression resulted in a 2.2–
fold to 15.7-fold increase, with a mean (SD) overexpression
of 10.08 (6.0). However, the D192G lamin C-FP typical
pattern was obvious in every experiment regardless of the
transfection efficiency or the overexpression levels.
Importantly, the presence of these giant spots in the D192G
lamin C-FP transfected cells could not be explained by a
higher rate of expression of the mutated than the wild type
lamin C-FP, as the transfection ratio of mutated versus wild
type was not different from 1 (1.15 (0.07)).

In order to mimic a heterozygous state, we coexpressed
wild type and the D192G lamin C. Coexpression only partly
restored the wild type phenotype (fig 3B), with approxi-
mately 50% of cells displaying a wild type phenotype (fig 3D).
Wild type and mutated lamin C co-distributed in the
nucleoplasmic foci (fig 3B). Transient expression of the
R541S lamin C-FP led to an intermediate phenotype with
fewer and more spherical foci than the wild type phenotype,
the number of cells displaying a unique spot being about 40%
(fig 3A, C). Coexpression of wild type and the R541S lamin C
completely restored the wild type phenotype (fig 3D).
Overexpression of the R190W and Y481stop lamin C did
not result in a specific phenotype (data not shown).

Figure 3  (A) COS7 cells transfected with fluorescently tagged wild type (WT) lamin C, D192G lamin C, and R541S lamin C showing the location of
the lamin C (red) within the nucleus visualised by Hoechst 33258 dye (blue). (B) COS7 cells co-transfected with equimolar amounts of fluorescently
tagged D192G (red) and wild type lamin C (yellow green) showing their co-location within the nucleus. Ratio of fluorescence was approximately 1. (C,
D). Number of spots per transfected cells or cells co-transfected with wild type and mutant lamin C. Data are presented as mean (standard error)
calculated from at least three independent experiments. \( \chi^2 \) tests showed significant differences across genotypes (p < 0.0001) for single transfection
experiments (C). The number of spots was significantly different in cells coexpressing D192G and WT lamin C compared with cells expressing WT
lamin C (p < 0.0001), and in cells coexpressing D192G and WT lamin C compared with cells co-expressing R541S and WT lamin C (p < 0.0001). The
number of spots was not significantly different in cells co-expressing R541S and WT lamin C compared with cells expressing WT lamin C (p > 0.1).
Black bars, number of cells with <3 spots; grey bars, number of cells with >3 spots.

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As previously reported for wild type lamin C-FP, wild type or D192G lamin C-FP foci did not disturb the distribution of endogenous lamins or emerin on the nuclear rim as detected by immunofluorescence (fig 4A–C). However, a readily detectable fraction of the endogenous lamin is recruited at the periphery of the foci (fig 4A, B).

It has been suggested that lamin A plays a central role in tethering lamin C to the nuclear envelope. We tested whether restoration of the stochiometry, through co-transfecting lamin A and C, leads to a relocalisation of lamin C-FP to the nuclear envelope. As shown in fig 5, co-transfection of wild type prelamin A and lamin C resulted in the redirection of lamin C-FP to the nuclear envelope.
of lamin C-FP to the nuclear envelope and the reduction of the granular aspect of the nucleoplasm in a significant proportion of the cells. By contrast, this phenomenon was never observed when both the D192G prelamin A and D192G lamin C were co-transfected (fig 5). The D192G lamin C speckles surrounded by the D192G lamin A tend to merge with the nuclear envelope at fusion points where an enrichment of lamin A was observed. However, the D192G lamin C was never identified in the lamina.

**Effect of the D192G LMNA mutation on SUMO1 distribution pattern**

Previous studies have proposed a role for internal lamin speckles in controlling gene expression and demonstrated that the reorganisation of lamin speckles into enlarged foci is correlated to inhibition of transcription. As major cell regulatory systems targeted by sumoylation are regulation of chromatin organisation and gene expression, we determined whether the normal distribution of SUMO1 was conserved in the presence of mutated lamin C. To this end, we co-expressed wild type or mutated lamin C and SUMO1 or SUMO1ΔC5 in COS7 cells. Western blotting quantification of endogenous versus transfected SUMO1 showed that the overexpression was around fivefold. SUMO1ΔC5 is a mutant form of SUMO1 lacking the C-terminal diglycine motif that is essential for covalent modification by SUMO1. As shown in fig 6A, SUMO1-FP was normally distributed in cell nucleus. When SUMO1-FP was co-transfected with the D192G lamin C-FP, SUMO1-FP appeared to be recruited and to concentrate in the middle of giant rounded lamin C spots (fig 6A). Co-expression of SUMO1-FP and R541S lamin C-FP resulted in a phenotype indistinguishable from the wild type phenotype (data not shown). By contrast, the SUMO1ΔC5-FP pattern remained diffuse in presence of both wild type and mutated lamin C (fig 6B). Therefore, the specificity of the dot-like staining of SUMO1-FP in the presence of the D192G lamin C seemed to be dependent on the sumoylation function of SUMO1.

**DISCUSSION**

By screening DNA from 92 DCM probands, we found four mutations confirming that LMNA mutations occur in a significant proportion (4%) of DCM patients. Although it has been suggested that LMNA mutation carriers with symptomatic DCM are characterised by poor prognosis, more than 18% of the adult carrier population we studied was unaffected. While the R190W LMNA mutation was previously reported in two related patients in their forties with DCM associated with conduction system defect, and in four related patients including one with isolated left ventricular noncompaction, to our knowledge the D192G, Y481stop and R541S mutations have not been reported elsewhere. However, an Y481H LMNA mutation was found to be associated with autosomal dominant limb girdle muscular dystrophy with cardiac conduction block. Mutations in the Arg541 codon were reported to be associated with Emery-Dreifuss muscular dystrophy (R541H) and DCM with conduction defects (R541C). Altogether, these results confirm that, despite DCM being considered a monogenic disorder, additional genetic and/or environmental factors contribute to the clinical phenotype.

Previous studies have reported nuclear membrane damage, such as focal disruptions, blebs, and nuclear pore clustering in myocytes from DCM patients with LMNA mutations. Similar morphological alterations of the nuclear membrane have been reported in different tissues from LMNA mutation carriers who are affected by other diseases, and in animal models. Based on these findings, it was postulated that the cardiomyocytes from the two end stage DCM patients carrying LMNA mutations would also demonstrate indications of nuclear membrane damage. Surprisingly, while heart tissue sections from the D192G LMNA carrier revealed...
marked nuclear abnormalities, cardiomyocytes from the patient carrying the R541S mutation presented only modest and non-specific nuclear membrane alterations. It is clear from these observations that direct correlations between any of the morphological changes and the mutations cannot be made.

In order to unravel the observed cardiac phenotypes, we expressed *LMNA* mutations in a cellular model and performed live cell analysis. While both phenotypes induced by overexpression of the R190W and Y481stop were indistinguishable from the wild type, overexpression of the D192G lamin C induced a reorganisation of the lamin speckles into a unique and giant round spot in the vast majority of cells, despite expression level of the D192G lamin C being similar to the wild type. When the R541S lamin C was expressed, the phenotype was intermediate with fewer spherical foci than the wild type phenotype. *LMNA* mutations resulting in punctuate distribution across the nuclear surface of lamin A and C have been previously reported in vitro,28 29 and in patient fibroblasts.14 30

To mimic a heterozygous state, we co-transfected the mutated lamin C with the wild type, and demonstrated that the rearrangement of lamin speckles was partially (D192G) or fully (R541S) reversed. The rescue of the wild type phenotype for R541S may explain why the patient carrying the R541S heterozygous mutation displays non-specific

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**Figure 6**  COS7 Cells co-transfected with fluorescently tagged lamin C (red) and (A) SUMO1 or (B) SUMO1 ΔC5 (yellow green). (A) Presence of wild type lamin C did not alter SUMO1 distribution (small dots within and nearby the nucleus), while co-transfection with D192G lamin C shows recruitment of SUMO1 within the lamin C spots. (B) No difference is observed in the localisation of SUMO1 ΔC5 in cell transfected with wild type lamin C or D192G lamin.
cardiac ultrastructural changes. It cannot be excluded that this patient carries a mutation in another DCM gene. By contrast, the discrete giant lamin speckles seen with the D192G lamin C persisted even in the presence of D192G lamin A, suggesting that those mutants are assembly incompetent. The lamin A tended to form a halo around the D192G lamin C speckles, which merged with the nuclear envelope at lamin A enrichment points. This lamin A halo was previously observed when cells were transfected with the N195K, E358K, and M371K mutant lamin A alone; here we show that the mutant lamin C is trapped inside. As a consequence of compromised lamina integrity observed in vitro, the nuclear envelope may become more fragile. This may result in the ultrastructural damage of the nuclear envelope, including the loss of the nuclear membrane in the heart tissue of patients carrying such mutations.

In vitro and in vivo interactions between A-type lamins and transcription factors have been reported. In lamin A/C deficient mice that develop DCM, alterations in nuclear architecture are associated with reduced PPARγ expression, a lack of hypertrophic gene activation, and decreased SREBP1. Given those data and our results, the speckle formation induced by mutated lamin A/C may result in “freezing” of the cells in a transient and/or a pathological phase that corresponds to a deregulated transcription state.

In conclusion, we showed an absence of specific phenotype for the R541S LMNA mutation both in vivo and in vitro. These findings question the supposition that the R541S LMNA mutation is the primary genetic defect responsible for the development of heart failure in such patients and therefore, raises concerns regarding the reliability of genetic counselling to patients and their families. By contrast, the D192G LMNA mutation is associated in vivo with dramatic morphological changes of the cardiomyocyte nucleus and in vitro with total disorganisation of type A lamins; this lamin mutant trapped SUMO1 modified proteins presented in the nucleus. Therefore, our hypothesis is that LMNA mutants leading to the formation of intranuclear speckles induce not only nuclear envelope structural damage, but also the deregulation of cellular functions controlled by sumoylation, such as transcription, chromosome organisation, and protein and RNA nuclear trafficking.

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