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Changes in splicing factor expression are associated with advancing age in man

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Abstract

Human ageing is associated with decreased cellular plasticity and adaptability. Changes in alternative splicing with advancing age have been reported in man, which may arise from age-related alterations in splicing factor expression.

We determined whether the mRNA expression of key splicing factors differed with age, by microarray analysis in blood from two human populations and by qRT-PCR in senescent primary fibroblasts and endothelial cells. Potential regulators of splicing factor expression were investigated by siRNA analysis.

Approximately one third of splicing factors demonstrated age-related transcript expression changes in two human populations. Ataxia Telangiectasia Mutated (ATM) transcript expression correlated with splicing factor expression in human microarray data. Senescent primary fibroblasts and endothelial cells also demonstrated alterations in splicing factor expression, and changes in alternative splicing. Targeted knockdown of the *ATM* gene in primary fibroblasts resulted in up-regulation of some age-responsive splicing factor transcripts.

We conclude that isoform ratios and splicing factor expression alters with age *in vivo* and *in vitro*, and that ATM may have an inhibitory role on the expression of some splicing factors. These findings suggest for the first time that ATM, a core element in the DNA damage response, is a key regulator of the splicing machinery in man.

Keywords

Ageing; Splicing; SRSF; hnRNP; Human

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Conflict of interest statement

The authors report no conflict of interest.

1. Introduction

Ageing is characterised by a generalised decrease of plasticity of cellular processes within tissues, including in tissue repair, immune repertoire and cognitive function (Wang et al., 2011; Asumda and Chase, 2011). Ageing is also associated with many common chronic diseases in the human population (Harman, 1991). The ageing process is however complex and heterogeneous, with some people surviving free of disease until advanced ages, whilst others become prematurely frail. Genome-wide association studies (GWAS) have identified variants that account for approximately one third of the variability in lifespan, so other factors must underpin the differences between people in how well they age (Harries et al., 2012a). Genomics, rather than genetics, may prove a key focus to identify the mechanisms involved in determining successful ageing (Kulminski and Culminskaya, 2013). Accordingly, gene expression analyses have already proven useful in the study of some age-related conditions such as cognitive impairment and decrease in muscle function in the elderly (Pilling et al., 2012; Harries et al., 2012b).

In a recent large-scale human population based study, we identified that a focussed set of transcripts and biological pathways show robust alterations in expression in leukocytes *in vivo* with advancing age, and that the majority of these pathways control messenger RNA splicing processes (Harries et al., 2011). These changes were also accompanied by alterations in the ratios of alternatively expressed transcripts. Deregulation of splicing with age has also been suggested to occur in some animal species (Yannarell et al., 1977; Meshorer and Soreq, 2002). In man, alternative splicing of the *LMNA* gene has been implicated in human ageing models; Mutations in *LMNA* that cause the skipping of exon 11 result in the monogenic Hutchinson Gifford Progeria syndrome (Cao et al., 2011), but low levels of the progerin isoform, which increase with age, have also been demonstrated in older humans (Graziotto et al., 2012). Other than this observation, there has to date been little systematic evaluation of the effect of ageing on the milieu of splicing variants in other genes.

Messenger RNA (mRNA) splicing is a co-transcriptional process where the initial RNA transcript (the hnRNA or primary RNA transcript) is modified to remove the non-coding introns, and to include the vital 5′ and 3′ regulatory regions (Cartegni et al., 2002; Dujardin et al., 2013). Up to 95% of all genes are alternatively spliced, enabling one gene to code for multiple proteins, which can have different spatial or temporal expression profiles, and differing or even opposing functions (Pan et al., 2008). The consequence of alternative splicing is to enhance the diversity and complexity of the proteome (Graveley, 2001) and to help cells specialise for tissue-specific functions. Alternative splicing also influences the ability of an organism to react and adapt to its environment; it is therefore a major player in the determination of cellular plasticity. Alternative splicing, like constitutive splicing, is regulated by a series of conserved, core, regulatory sequence elements; the splice donor site, the splice acceptor site, the branch site and the polypyrimidine tract. However, for alternatively spliced transcripts, the consensus sequence is essential, but not always sufficient, for splice site usage. Auxiliary splice site elements (exon and intron splicing enhancers and silencers) are often required to add an additional layer of regulation (Cartegni et al., 2002). These regulatory elements can work by binding a set of splicing regulator

proteins; serine/arginine-rich splicing factor (SRSF) proteins, which promote exon inclusion and heterogeneous nuclear ribonucleoproteins (hnRNPs), which usually inhibit cassette exon inclusion (Smith and Valcárcel, 2000). The role of SRSFs and hnRNPs are not exclusive to splicing, SRSFs are involved in several other features of mRNA biology including translation, nonsense-mediated decay and mRNA export (Kervestin and Jacobson, 2012; Hocine et al., 2010). These proteins can act antagonistically and in some cases can have the opposite effects (Caceres and Kornblihtt, 2002).

There are several factors which could influence splice site usage during human ageing. It is possible that accumulated DNA damage to the sequences of the core or auxiliary splicing factor binding sites has the potential to cause splicing alterations with age, but such effects would be cell-specific and heterogeneous, and therefore unlikely to produce a measurable difference in the level of any particular isoform in a mixed population of cells. However, our previous data suggests that age-related deviations from normal splicing patterns may instead be due to altered expression of the factors (splicing enhancers and silencers) that bind these sequences (Harries et al., 2011). Therefore, in this study, we aimed to use a combination of *in vivo* mRNA expression analyses in two populations, *in vitro* cell senescence experiments and targeted knockdown of key genes to identify potential molecular mechanisms that could affect the mRNA expression of core and regulatory splicing factors in man.

2. Materials and methods

2.1. Study cohorts

We used microarray data from two separate human populations, the InCHIANTI study and the San Antonio Family Heart Study. The InCHIANTI study is a large longitudinal study of individuals living in the Chianti region of Tuscany (Ferrucci et al., 2000). The microarray dataset comprises expression data on 16,571 transcripts expressed in peripheral blood from 695 subjects aged 30–104 years (Harries et al., 2011). Blood was collected into PAXgene tubes (BD Biosciences), and extracted using the PAXgene blood RNA kit (Qiagen, Paisley, UK). Microarray analysis was carried out on the Illumina HT-12 array (Illumina, San Diego, USA). The second population, the San Antonio Family Heart Study (SAFHS), comprises 1238 individuals of Mexican ancestry aged from 15 to 94 years (Goring et al., 2007), with publicly-available expression data from 14,727 transcripts in isolated lymphocytes. Participant characteristics have been described (Harries et al., 2012a; Mitchell et al., 1994). All microarray experiments and analyses complied with MIAME guidelines.

2.2. Choice of splicing factor and splicing regulator transcripts for analysis

We identified transcripts encoding components of the core splicing machinery and known splicing regulators by reference to splicing factor databases (http://rulai.cshl.edu/cgi-bin/SpliceFac/). The presence of each transcript in the microarray expression data from each population was assessed by a search of Human Genome Organization Gene Nomenclature Committee (HGMC) gene identifiers (www.genenames.org) prefixed with the following character strings; “HNRNP”, “IMP”, “LSM”, “RRP”, “SF3”, “SNRNP”, “U2” or “SFR”, indicative of core or auxiliary splicing factors. We identified a list of 71 splicing factors or regulators for analysis, for which microarray expression data were available in the

InCHIANTI dataset. The target list comprised of hnRNPs (usually splice site inhibitors), SRSFs (usually splice site activators), as well as some of the core spliceosomal proteins involved in constitutive splicing. Of the 71 splicing-related transcripts expressed in the InCHIANTI data, 55 transcripts were also represented in the SAFHS microarray data (see supplementary Table 3 for a full list of genes included in each analysis and supplementary Table 4 for transcript and microarray probe identifiers).

2.3. Correlation of splicing factor expression with age in human populations

Statistical associations between the expression level of each test transcript and age were tested using fully adjusted multivariate adjusted linear regression models (R Statistical Computing Language v2.14.0), with logged expression data as the outcome variable. InCHIANTI regression models were adjusted for sex, waist-circumference (cm), social-status (highest education level attained, five categories: none, elementary, secondary, high-school and university/professional), pack-years smoked (five categories: none, <20 years, 20–39 years, >40 years, and missing), study site ([Greve] or [Bagno a Ripoli]), batch effects (amplification and hybridization), and the proportion of leukocyte sub-type in the blood (lymphocytes, monocytes, neutrophils and eosinophils). SAFHS regression models were adjusted for sex and smoking-status, as data on these factors alone were available. We accounted for multiple testing using false discovery rate (FDR – package “fdrtool” (Strimmer, 2008)) adjusted *p*-values (*q*-values), using the conventional cut-off of $q <$ for statistical significance. Separate regression screens were performed for each cohort.

2.4. Assessment of core or regulatory role of differentially expressed splicing factors

The splicing factors which were analysed with age in the INCHIANTI and SAFHS population were divided into two groups, those demonstrating a significant association with age in the fully adjusted model ($q = <0.05$), and those not. Splicing factors were identified as being “core” (i.e. part of the constitutive spliceosome) or “regulatory” (i.e. hnRNP-related splicing inhibitory transcripts or SRSF-related activating transcripts). Differences in the distribution of “core” and “regulatory” splicing factors between the ‘significant’ and ‘non-significant’ transcript sets were assessed using χ^2 test.

2.5. In vitro senescence

2.5.1. Senescence of human primary cell lines in vitro—To examine the relationship between splicing factor expression and cellular ageing without any of the confounding factors associated with epidemiological studies, we carried out *in vitro* senescence of two primary cell types, human aortic endothelial cells (HAOEC) and normal human dermal fibroblast cells (nHDF), which have been reported as undergoing cellular senescence by different processes (Shelton et al., 1999). Cells were purchased from Promocell, Heidelburg, Germany. Endothelial cells were isolated from the human abdominal and thoracic aorta and fibroblasts were derived from human skin taken from the thigh. Cells were tested for the presence of mycoplasma at source and also for cell type specific markers to confirm identity and both cell types were at passage 2 after thawing which corresponds to less than 15 population doublings. Three independent cultures underwent *in vitro* senescence by repeated culture until growth arrest as biological replicates for each cell type. Culture

media included 1% penicillin and streptomycin and a cell-specific supplement mix: fibroblasts – foetal calf serum 0.03 ml/ml, recombinant fibroblast growth factor 1 ng/ml and recombinant human insulin 5 μg/ml; and endothelial cells–foetal calf serum 0.05 ml/ml, endothelial cell growth supplement 0.004 ml/ml, epidermal growth factor 10 ng/ml, hydrocortisone 1 μg/ml, heparin 90 mg/ml). Cells were cultured in humidified incubators at 37 °C and 5% CO_2. Cells were cultured until they reached 80–90% confluence serially until the cells reached growth arrest.

2.5.2. Biochemical, molecular and morphological assessment of cell senescence—Cell senescence was assessed by population doubling times, qualitative assessment of morphological changes and staining for senescence-associated β-galactosidase activity (Sigma Aldrich, UK) following the methods previously described (Dimri et al., 1995). Expression of the *CDKN2A* and *VEGFA* genes was also measured by quantitative real-time PCR (qRT-PCR), by TaqMan Low Density Array (TLDA) on the ABI Prism 7900HT platform, as molecular markers of cell senescence (assay identifiers available on request). Real-time PCR conditions are described in detail below. Cells were considered senescent two to four passages prior to absolute growth arrest, when molecular and biochemical assessment indicated senescence, but whilst the cultures were still capable of division and did not exhibit signs of cellular stress.

2.5.3. Expression profiling of splicing factor expression in in vitro senesced cells—Splicing factor transcripts were selected for inclusion on the TLDA card based on evidence from our microarray screen of age-associated expression. Most of the transcripts selected encoded splicing regulatory proteins (*hnRNPA0, hnRNPA1, SRSF1, SRSF6*) although some core spliceosomal proteins (including *LSM14A*, *IMP2* and *LSM2*) were included. A full list of the assay identifiers and the reference sequences of the mRNAs they detect is given in supplementary Table 5. The endogenous controls were *IDH3B, GUSB and PPIA* which were selected based on empirical evidence for a lack of association with age in the microarray data (Harries et al., 2011).

RNA was extracted from endothelial cells and fibroblasts at early and late passage using the Qiagen RNA extraction kit (Qiagen, West Sussex, UK), following the manufacturer's instructions. Total RNA (100 ng) was reverse transcribed in 20 μl reactions using the Superscript III VILO kit (Life Technologies, Foster City, USA) prior to TaqMan Low Density Array (TLDA) analysis. Quantitative RT-PCR reactions were performed on the ABI 7900HT platform (Life Technologies, Foster City, USA). Cycling conditions were 50 °C for 2 min, 94.5 °C for 10 min and 40 cycles of 97 °C for 30 s and 57.9 °C for 1 min. The reaction mixes included 50 μl TaqMan® Fast Universal PCR Mastermix (no AmpErase® UNG) (Life technologies, Foster City, USA), 30 μl dH_2O and 20 μl cDNA template. 100 μl reaction solution was dispensed into the TLDA card chamber and centrifuged twice for 1 min at 1000 rpm to ensure distribution of solution to each well. The gene expression of each sample was measured in triplicate in "young" and "old" cells. The comparative Ct technique was used to calculate the expression of each test transcript (Pfaffl, 2001). Expression was assessed relative to the mean of the three endogenous controls *IDH3B, PPIA and GUSB* with normalisation back to the mean value for the transcript in the early passage cells in each

case. Mann–Whitney-*U* analysis was used to test statistical significance of expression differences between early and late passage cells. All statistical analyses were carried out using STATA v12.0 (StataCorp LP, Texas, USA).

2.5.4. Analysis of alterations in isoform expression for known ageing related genes—As a measure of downstream consequences of alterations in splicing factor expression in senescent cells, we measured the ratio of alternatively expressed isoforms of a small panel of genes known to be important in ageing or to have altered expression with age. These included the known age-related genes *CDKN2A* (Krishnamurthy et al., 2006), *VEGF* (Ryan et al., 2006), *TERT* (Tomas-Loba et al., 2008), as well as the *EFNA1, GPR18* and *VCAN* genes, which we demonstrated to exhibit alterations in isoform ratios during human ageing in peripheral blood in our previous study (Harries et al., 2011). Transcript and assay details are given in supplementary Table 6). Expression profiling was carried out on the TLDA platform as described earlier, with isoform ratio changes examined for statistical significance by Mann–Whitney-*U* analysis of the isoform expression ratios in 'young' and 'old' cells.

2.6. Epidemiological investigation of potential regulators of splicing factor expression

To identify other transcripts in the genome with a potential role in the regulation of splicing factor expression, we carried out a transcriptome-wide, fully-adjusted multivariate regression analysis to identify transcripts associated with age itself, and also with the expression of two of the most deregulated splicing regulator transcripts *SRSF6* and *hnRNPA0*. Models were adjusted as described in the sections above.

2.7. Assessment of ATM transcript levels in senescent cells

We measured the expression levels of the ATM transcripts encoding a key component of the DNA damage response in both endothelial cells and in fibroblasts by qRT-PCR. cDNA was produced as described above, using the Superscript III VILO kit (Life Technologies, Foster City, USA) and qRT-PCR reactions were carried out in triplicate in 96 well plates in 10 μl volumes. Reactions contained 5 μl TaqMan Fast Universal Mastermix (no AMPerase) (Life Technologies, Foster City USA), 0.25 μM probe and 2 μl cDNA reverse transcribed as above in a total volume of 10 μl. PCR conditions were a single cycle of 95 °C for 20 s followed by 50 cycles of 95 °C for 1 s and 60 °C for 20 s. The probes to each transcript comprised a pre-validated off-the-shelf assay provided by Life Technologies (Foster City USA); Assay identification number available on request). The relative expression level of each transcript was then determined relative to the *GUSB* and *GAPDH* endogenous control and normalised to the level of each transcript in the low passage cells.

2.8. In vitro knockdown of ATM expression in early passage fibroblasts

The knock-down of ATM in passage 9 fibroblasts was carried out using Silencer® Select siRNAs from Ambion® (Life Technologies, Foster City, USA) s57221 for knockdown of ATM reference sequence NM_000051.3 (silencer s577300). Silencer® select negative and GAPDH positive control siRNA were used to determine knock down as well as a normal control sample. We performed the knockdowns with NHDF Nucleofector[1] Kit (Lonza, Switzerland) as per the manufacturer's instructions with 30 mM, 100 mM or 300 mM of

Silencer® siRNA to optimise gene knockdown. Two separate siRNAs were used for validation.

$5 \times 10^{+4}$ fibroblasts cells were resuspended in 100 μl nucleofection solution (Lonza, Switzerland) and fibroblasts were transferred into the nucleofection cuvette containing scrambled siRNA, GAPDH siRNA or no silencer. Following the short electroporation (programme P-022 for Nucleofector® device), 100 μl culture medium was added to the electroporated fibroblasts, transferred into a 6-well plate and incubated for 48 h. RNA was extracted from electroporated fibroblasts using the *mir*Vana™ miRNA Isolation Kit (Life Technologies, Foster City, USA) following the manufacturer's instructions. A Nanodrop spectrophotometer (Thermo Scientific Ltd, Wilmington, USA) was used to quality control RNA and RNA was only accepted for analysis if the concentration was >30 ng/μl. Total RNA (100 ng) was reversed transcribed in 20 μl reactions using the Superscript III VILO kit (Life Technologies, Foster City USA), according to the manufacturer's instructions. *ATM* knockdown in the primary fibroblast cells was assessed by measuring the levels of *ATM* probes Hs00175892_m1 and Hs01112307_m1 which detect *ATM* reference sequence NM_000051.3 (life technologies, Foster City, USA). The expression of *ATM* siRNA treated samples were assessed (as per the assessment of ATM expression in senescent cells) relative to the *GAPDH* endogenous control and normalised to the level of *ATM* transcripts in the negative and normal controls. Knockdown of the ATM transcripts was confirmed by qRT-PCR carried out on each sample in triplicate using probes specific to the *ATM* transcript on the ABI Prism 7900HT platform (Life Technologies, Foster City, USA).

2.9. Assessment of splicing factor expression following siRNA-mediated knockdown of ATM transcripts

RNA from the cells in which we had confirmed *ATM* transcript knockdown was then used to assess splicing factor mRNA transcript expression using the TLDA approach as described in previous sections. Splicing factor transcript expression results in from the *ATM* siRNA treated fibroblasts was compared with both untreated cells, and with cells treated with the scrambled control to ensure that any differences noted derived from the specific knockdown and not from manipulation of cells. Differences in splicing factor mRNA expression between ATM knockdown and control cells were examined for statistical significance using Mann–Whitney-*U* test.

3. Results

3.1. Differences in splicing factor expression are associated with age in two human populations

We identified that 27/71 (38%) splicing proteins tested were significantly associated with age following adjustment for multiple testing in the INCHIANTI microarray dataset, with 15/55 (29.4%) transcripts being significantly associated with age in SAFHS (Table 1). Fig. 1 shows histograms for the most age-associated splicing transcripts in the InCHIANTI and SAFHS cohorts. All age-associated transcripts were down-regulated with the exception of *SFRS14*, *SRSF3* and *SRSF11* in the InCHIANTI dataset and *SRSF14*, *RALY* and *HNRNPAB* in SAFHS. 51 transcripts were common to both datasets (Table 1 and

supplementary Table 3), of which 9 (*SRSF1*, *SRSF6*, *LSM5*, *LSM2*, *SRSF14*, *SF3B1*, *HNRNPAB*, *HNRNPD* and *HNRNPH3*) were significantly associated with age in both populations (Table 1). Fig. 2 shows the overlap of the age-associated splicing factor expressions in the InCHIANTI and SAFHs cohorts.

3.2. Splicing regulator transcripts are more likely than core splicing factor transcripts to be associated with age in the InCHIANTI dataset

Transcripts coding for regulators of splicing were significantly more likely to be associated with age than core spliceosomal RNAs in the InCHIANTI cohort. In the significantly associated set of transcripts, 74% of mRNA transcripts coded for splicing regulatory proteins rather than core spliceosomal components, whereas in the non-significant group, only 34% of transcripts encode splicing regulators, which was significant at the level of $p = 0.001$ as assessed by χ^2 squared test. However, in the SAFHS microarray data (with 55 vs. 71 genes testable in InCHIANTI), we did not note any preponderance of splicing regulator transcripts in the age-associated dataset ($p = 0.907839$).

3.3. Primary fibroblasts and primary endothelial cells undergo replicative senescence at different rates

We achieved molecular, biochemical and cell morphology parameters consistent with cellular senescence for both fibroblasts and endothelial cells *in vitro* (Table 2). Senescence was accompanied by increased β-galactosidase staining, a biochemical marker of cellular senescence (Dimri et al., 1995), and alterations in morphology and population doubling time were recorded in both cell types (Table 2). Fibroblasts ceased growth at passage (p) 22, corresponding to approximately 53 cell doublings whilst endothelial cells ceased growth at (p) 15, corresponding to 35 cell doublings. Both cell types had a slower population doubling time with increasing senescence, endothelial cells reached confluence within 3 days in the early passages increasing to 10 days in the latest passage and fibroblasts slowed from 4 to 27 days. Splicing factor and isoform expression was therefore assessed at p7: for both cell types (termed “young” cells): PD = 21 in endothelial cells and PD = 23 in fibroblasts, and at p13 (PD = 33) in endothelial “older” cells and p18 (PD = 45) in fibroblast “old” cells. We excluded terminally senescent cells from our analyses in order to ensure that we were examining senescence, rather than stress response changes. β-Galactosidase staining was visibly present in 68% of the endothelial cells at p13 and 58% of the fibroblasts at p18 (Fig. 2).

3.4. Splicing factor expression demonstrates age-related changes in senescing primary cell cultures

We identified that the expression level of all 20 of the selected splicing regulatory factors were significantly associated with age in fibroblasts (see Table 3). All splicing factors and regulators were expressed at significantly lower levels in older fibroblasts with the exception of *HNRNPUL2*, *PNISR* and *SF3B1*, which demonstrated higher expression in older cells. Senescence-associated differences in the expression of splicing factor transcripts was evident in endothelial cells, although not as comprehensively; only 9 of the 20 transcripts analysed were associated with age in endothelial cells, and several of these were positively, rather than inversely, correlated with age (Table 3). Representative box plots of the top three most

associated splicing factors for fibroblasts (*PNISR*, *hnRNPA1* and *hnRNPA2B1*) and endothelial cells (*hnRNPK*, *hnRNPM* and *hnRNPUL2*), are given in Fig. 3.

3.5. Cellular senescence is associated with changes to the ratio of alternatively expressed isoforms for key ageing genes

For the genes selected to assess downstream consequences of age-related changes to splicing factor gene expression, we were able to detect expression for 3 genes (*CDKN2A*, *VCAN* and *VEGFA)* in fibroblasts and 5 genes (*CDKN2A*, *VCAN1, GRP18, EFNA1* and *VEGFA*) in endothelial cells, although different genes were expressed in each cell type. We identified age-related differences in the ratio of alternatively expressed isoforms in fibroblasts for the *CDKN2A* ($z = -3.811$, $p < 0.001$) and *VCAN* ($z = 4.157$, $p < 0.001$) genes, with isoform changes of *VEGFA* isoforms also trending towards significance (Table 4). The ratio changes for *CDKN2A* in aged fibroblasts were driven by an increase in expression of transcripts coding for the p16INK4a isoform, whereas changes for *VCAN* were driven by an increase in the expression regulation of the variant 4 isoform. In endothelial cells, we noted changes in the mRNA isoform ratios of a different set of transcripts; the largest ratio changes were apparent for the *VEGFA* gene ($z = -3.70$, $p < 0.001$) (Table 4), whereas changes in *CDKN2A* isoform expression between “young” and “old” endothelial cells were less apparent (Table 4). The ratio changes for *VEGFA* in endothelial cells do not rely on changes to one mRNA transcript, but both of those measured were down-regulated with age.

3.6. ATM may be a regulator of splicing factor expression in man

In the InCHIANTI human microarray data we identified 19 transcripts which were associated with age and also with the expression of two of the most age-deregulated splicing factors *SRSF6* and *hnRNPA0* (supplementary Table 1). Of these, only 3 transcripts; Ataxia telangiectasia mutated (*ATM*), SAFB-like (*SLTM*) and poly (A) binding protein interacting protein 2 (*PAIP2*) have any reported role in RNA biology (Tsai et al., 2012; Berlanga et al., 2006; Zhang et al., 2011). The transcript coding for ATM was considered of particular interest because of its role in the regulation of KH-type splicing (Zhang et al., 2011), its important role as part of the DNA damage response and its known activation in cell senescence (Suzuki et al., 2012). *ATM* expression in leukocytes was negatively correlated with advancing age in the InCHIANTI human data (coefficient −0.01402, FDR adjusted *q*-value = 1.9×10^{-5}). Conversely ATM expression in the older fibroblast cells *in vitro* was increased with age ($z = -3.046$, $p = 0.002$).

3.7. siRNA-mediated transcript knockdown of the ATM gene influences the expression of several splicing factors in primary fibroblasts

We undertook siRNA knock-down experiments of *ATM* expression in normal human dermal fibroblasts at passage nine. The positive control, an siRNA targeted against *GAPDH* produced an average of 75% reduction in *GAPDH* transcripts across 3 replicates. We achieved an average of 60% reduction in *ATM* expression in six biological replicates treated with an siRNA, in comparison to both untreated cells, and those treated with a scrambled siRNA (supplementary Table 2). RNA was extracted from the *ATM* siRNA-treated cells and the controls, and analysed for the expression levels of splicing factors by TLDA as described above. The expression of eight splicing regulatory factors were significantly increased in the

cells exposed to the *ATM* siRNAs; *SRSF3* ($z = -.3.49$, $z = <0.001$), *HNRNPD* ($z = -2.252$, $p = 0.0243$) *HNRNPA1* ($z = -2.483$, $p = 0.013$), *LSM14A* ($z = -2.078$, $p = 0.0377$), *SRSF1* ($z = -2.598$, $p = 0.0094$), *SRSF2* ($z = -2.021$, $p = 0.0433$), *SRSF7* ($z = -2.252$, $p = 0.0243$), and *TRA2B* ($z = -2.483$, $p = 0.013$) (Table 5). Representative graphs of the 4 transcripts showing the largest increases in expression following *ATM* siRNA treatment are shown in Fig. 4.

4. Discussion

In this study, we present the first evidence from human populations and from primary human cells ageing to senescence *in vitro*, that age-related deregulation of splicing patterns may be attributable to changes in the pool of core and regulatory splicing factors. Changes in splicing factor expression with age may thus be partially attributable to age-related changes in the expression of the Ataxia Telangiectasia Mutated (*ATM*) gene, which we have identified as a potential regulator of splicing factor expression for the first time. Changes to the normal splicing patterns are likely to have deleterious consequences for cell physiology, and may underlie some of the reductions in cellular adaptability and plasticity that occur during the ageing process (Fig. 5).

We found age-related differences in the milieu of splicing factors to be evident in expression array data from two separate human populations (InCHIANTI in whole blood white cell samples and SAFHS in isolated lymphocytes), and also in two primary cell lines of different lineages; fibroblasts and endothelial cells. It is of course not possible to infer activity of splicing factors from expression data alone, but our observation that the ratio of alternatively expressed transcripts also differ with age in blood, fibroblasts and endothelial cells indicates that the changes in splicing factor expression we note may have functional effect. The precise identity of splicing factors affected, and the direction of effect is not always consistent between different tissue types, indicating some degree of tissue specificity. This may also explain our observation that splicing factors associated with human ageing are more likely to be splicing regulators rather than core components in the spliceosome in whole blood (inCHIANTI dataset) than they are in isolated lymphocytes (SAFHS dataset). This may reflect differences in array coverage or it may reflect alterations in blood cell subtypes that are known to occur with age (Alam et al., 2012). Differences in the milieu of splicing factors between tissues is expected, since splicing patterns are known to be highly tissue specific, and the correct expression and regulation of specific genes is of paramount importance in controlling cellular identity. In primary fibroblasts, whole blood and leukocytes, most of the splicing factors we assessed demonstrated reduced expression with age, whereas in primary endothelial cells, both increases and decreases in splicing factor expression were noted. This is concordant with the differences in senescence processes previously reported between fibroblasts and endothelial cells (Shelton et al., 1999). One interesting feature between the datasets is the reduced expression of splicing activator transcripts (*SRSF* transcripts) in the fibroblasts and leukocytes and the increased expression of inhibitory splicing factors (*hnRNP* transcripts) in the endothelial cells, all of which are consistent with a decrease in splicing complexity. The alterations to the pool of *SRSFs* and *hnRNPS* we note could result in a decrease in the ability of senescent cells to regulate the inclusion of cassette exons. This is important since the inclusion of cassette exons containing

premature termination codons ("poison" exons) is an emerging mechanism of controlling gene expression; transcripts containing such exons are susceptible to degradation by the nonsense-mediated decay (NMD) pathway (Grellscheid et al., 2011). These changes therefore could contribute to the loss of adaptability and plasticity of cells and tissues with age and have further consequences such as the altered proportions and effectiveness of innate and adaptive immune cells associated with an impaired response of the immune system to vaccines in the elderly and an enhanced vulnerability to infections (Weinberger et al., 2008).

A search for transcripts linked not only to age, but also to levels of some of the most deregulated splicing factors (*SRSF6* and *hnRNPA0*) revealed the *ATM* gene as a potential regulator of splicing factor gene expression (see supplementary Table 2). ATM is a key regulator of the complex DNA damage response signalling pathway (Shiloh, 2006), where it binds directly to the site of double stranded DNA breaks and induces the activation of various cell cycle pathways including activation of *MYC*, *ARF*, *MDM2* and *p53* proteins in response to DNA damage (Campaner and Amati, 2012). DNA damage, caused by various intra- and extracellular insults, accumulates with age and has been identified as a major player in the ageing process alongside decreases in DNA repair capacity (Jackson and Bartek, 2009). Interestingly, we note that *ATM* levels are inversely correlated with age in leukocytes in population study, but positively correlated with age in the senescent fibroblasts. A decrease in levels of *ATM* transcripts with age is at first counterintuitive, but can be explained by the observation from publicly available microarray data that very high levels of *ATM* have been found in CD4 and somewhat lower levels in CD8+ve naïve T-cells (from a small sample of individuals, http://biogps.org/#goto=genereport&id=472), the proportions of which are known to decrease with age (Saule et al., 2006). Only by reference to a single population of cells from a single origin were we able to identify that levels of *ATM* transcripts actually increase, not decrease with age, as might be expected from a marker of accumulated DNA damage.

Our hypothesis that splicing factor expression may be attenuated during the ageing process in man by the expression of a potential regulator ATM is supported by our observation that targeted reduction of *ATM* transcript levels by siRNA in young (passage 9) fibroblasts results in the up-regulation of a subset of splicing factors. We elected to use young fibroblasts rather than senescent fibroblasts for several reasons. Firstly, senescent fibroblasts may have higher levels of other cellular stress proteins that may influence splicing factor expression, making it difficult to assess the effect of *ATM* knockdown alone. Senescent cells may also have accumulated mutations to the sequence elements required for regulation of expression. Finally, transfection of senescent fibroblasts is technically difficult, and may produce differences in gene expression profiles unrelated to the manipulation of *ATM* expression. Splicing factors demonstrating expression differences in response to targeted reduction of *ATM* expression include five of the 8 members of the *SRSF* family of splicing activators tested (*SRSF1*, *SRSF2*, *SRSF3*, *SRSF7* and *TRA2B*; see Table 5), which are known to be master regulators of alternative splicing (Ghosh and Adams, 2011). In contrast, only 2/8 of the *hnRNP* splicing inhibitory transcripts tested (*hnRNPA1* and *hnRNPD*) responded to *ATM* down-regulation by siRNA, indicating that some of the splicing inhibitors may be responsive to other factors. However, regulation of splicing factor

expression is complex and has previously been shown to be influenced by other factors such as the expression of the *MYC* gene (Das et al., 2012), which is known to be another part of the DNA damage response (Campaner and Amati, 2012). Our data indicate that *ATM* is a negative regulator of members of the *SRSF* group of splicing factors, which together with other splicing factors such as the hnRNPs play a role in the orchestration of alternative splicing. One potential explanation for this is the role of ATM in the biogenesis of microRNAs, which are mainly repressors of gene expression by mRNA degradation or translational inhibition (Zhang et al., 2011). The regulatory role of ATM in miRNA biogenesis is modulated by KH-type splicing regulatory protein (KSRP) (Zhang et al., 2011). Accordingly, it has subsequently been reported that *SRSF1* is regulated by *miR-505* and *miR-28* in mouse embryonic fibroblasts (Verduci et al., 2010). These findings indicate that investigation of *ATM*, *KSRP* and microRNA regulation is potentially a fruitful future avenue of study. Other future areas for consideration involve the targeted knockdown of multiple splicing regulators to observe effects on isoform expression. Splice site choice is dependent on the presence of multiple splicing regulators (SRSF proteins and hnRNPs), and effects on isoform expression may thus be difficult to assess from knockdown of a single regulator.

The strengths of our study are the combined use of both molecular epidemiological studies in two separate populations and mechanistic approaches in *in vitro* models of human cell ageing, to address the likelihood of changes in splicing factor expression during the ageing process. This allows us to use a ‘real-life relevant’ scenario in the form of the population studies, and also a more controlled analysis in a single homogeneous cell type, which is not subject to the same confounding influences and mix of tissue types that the epidemiological studies are. The use of cell model systems also allows intervention to assess causality and direction of effect. A further strength of our study arises from the fact that the two populations studied differ not only in their cell subtypes but also in their sample collection, storage and their analyses. Despite these differences we have found that the splicing factor milieu is associated with advancing age in both cohorts, despite the differences in experimental approach. This lends confidence to our results. It is also unclear whether fully senescent cells occur *in vivo* (Boisen and Kristensen, 2010). However, evidence from the Sedivy group indicates that a proportion of senescent cells are present in the tissues of ageing primates (Jeyapalan et al., 2007; Herbig et al., 2006), indicating that the use of replicative senescence is a reasonable model of ageing *in vivo*.

5. Conclusions

We report here the first human evidence that the differences to alternative splicing patterns that occur during the ageing process (Harries et al., 2011; Cao et al., 2011; Yannarell et al., 1977) may arise from mRNA expression changes to the milieu of core and regulatory splicing factors, and that a proportion of these are in turn regulated by the DNA damage response protein ATM. These data may represent a key link between age-related DNA damage and the processes of alternative splicing which direct adaptability, plasticity and cell identity. Alterations in alternative splicing may underpin both some of the heterogeneity of ageing in man and potentially some of the associations with age related diseases, many of which are highly dependent on correct splicing (Padgett, 2012; Wiszomirska et al., 2011;

Nelson and Keller, 2007). More work is now required to elucidate the mechanisms by which the composition of the splicing factor milieu is regulated and how this in turn may impact on human ageing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mad.2013.05.006.

Abbreviations

ATM	Ataxia Telangiectasia Mutated gene
siRNA	small interfering RNA
SRSF	SR splicing factor
hnRNP	Heterogeneous nuclear ribonucleoproteins
LMNA	Lamin A/C gene
qRT-PCR	quantitative real-time PCR
TLDA	TaqMan Low Density Array
RT-PCR	Reverse Transcription PCR

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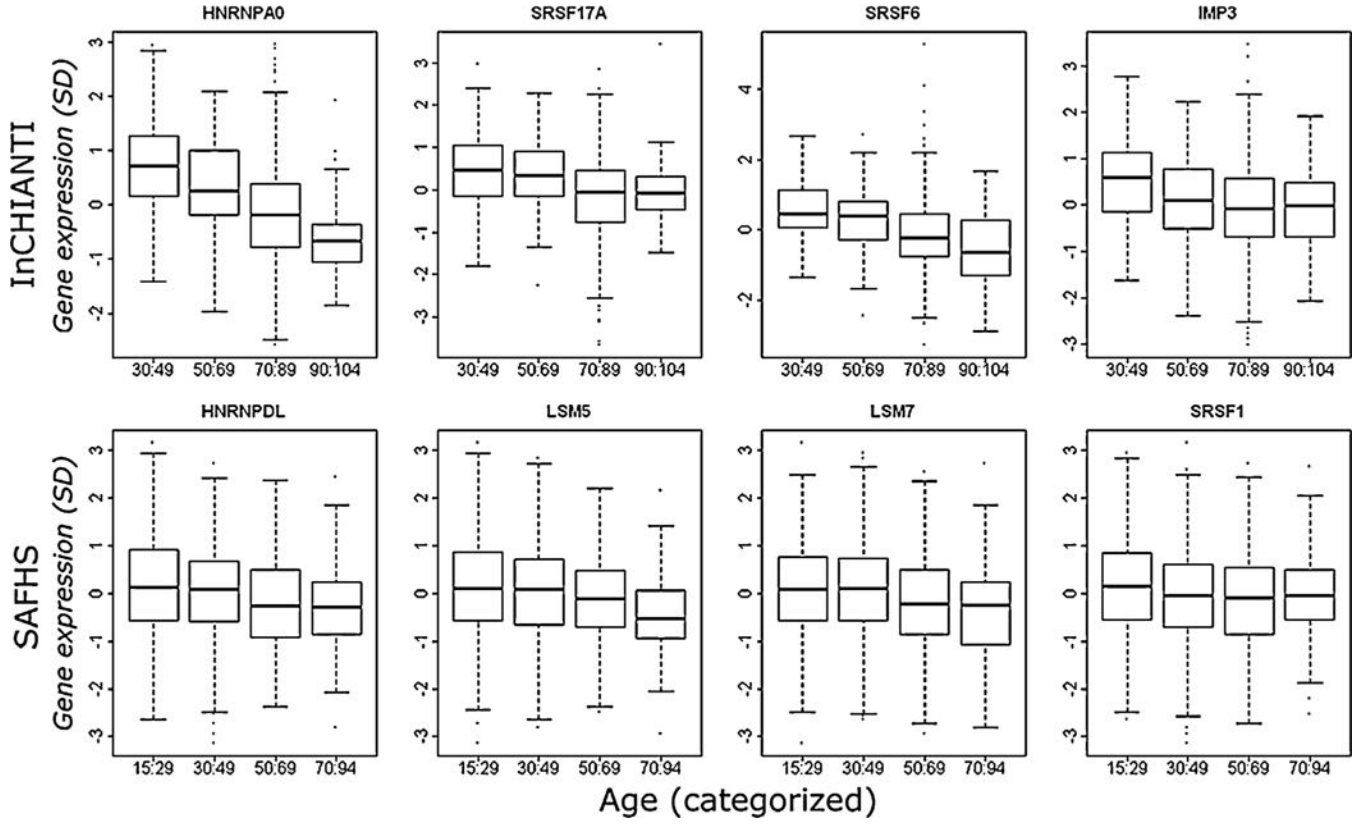

Fig. 1.

Box plots showing the relationship between gene expression levels and increasing age in the InCHIANTI and SAFHS populations. Standardised gene expression levels relative to the endogenous controls are shown on the *Y*-axis and the population age groups are shown on the *X*-axis. These are the top four splicing factor gene expression changes with age in the InCHIANTI and SAFHs populations.

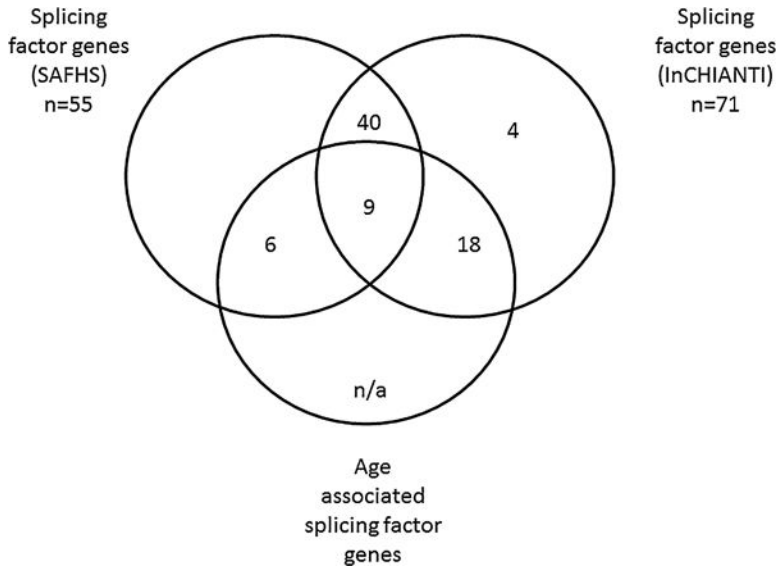

Fig. 2.

Venn diagram indicating gene expression transcripts associated and unassociated with age in the two population cohorts, SAFHS and InCHIANTI. The mRNA transcripts with the same associations in both populations are also identified.

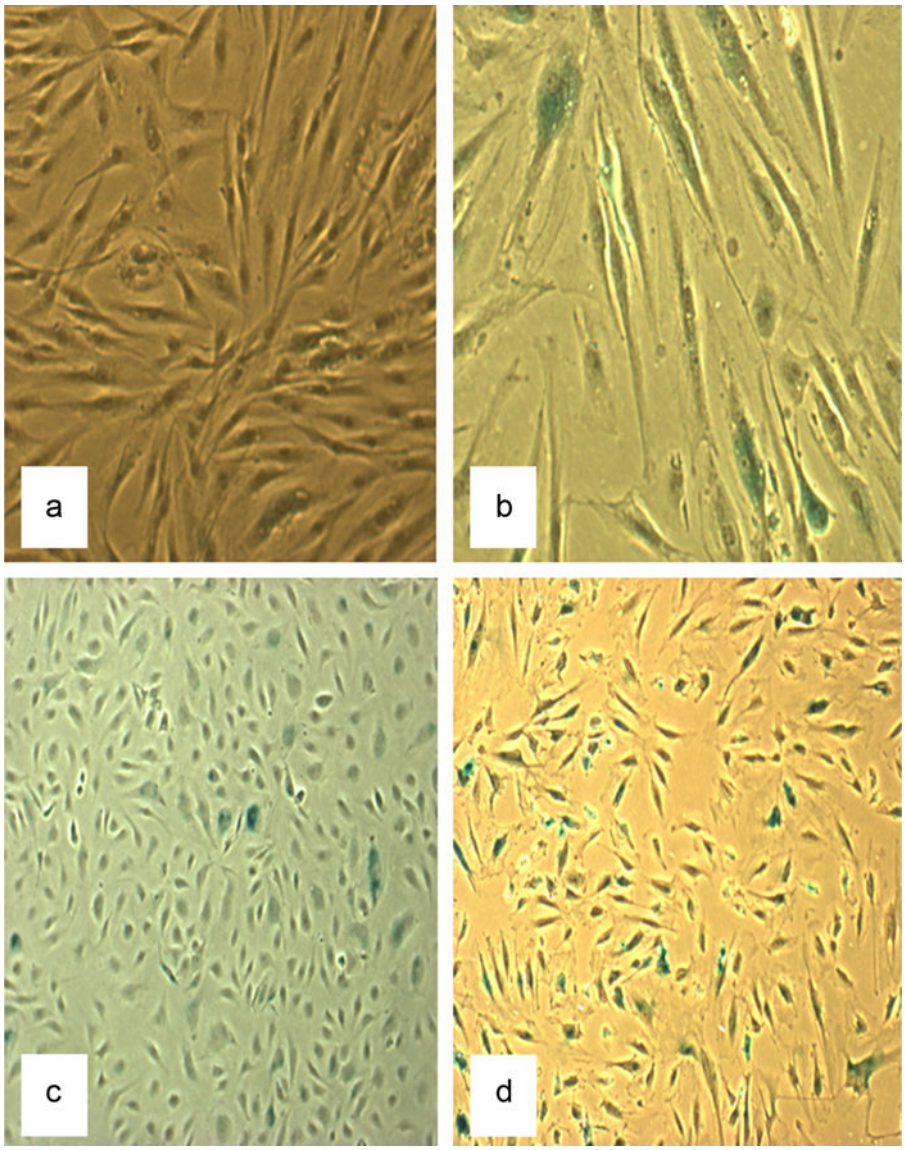

Fig. 3.

The figures show β-galactosidase activity as a marker of cellular senescence. A = fibroblasts at low passage (p6; 5% staining), B = fibroblasts at high passage (p20; 58% staining), C = endothelial cells at low passage (p6; 7% staining) and D = endothelial cells at high passage (p13; 68% staining).

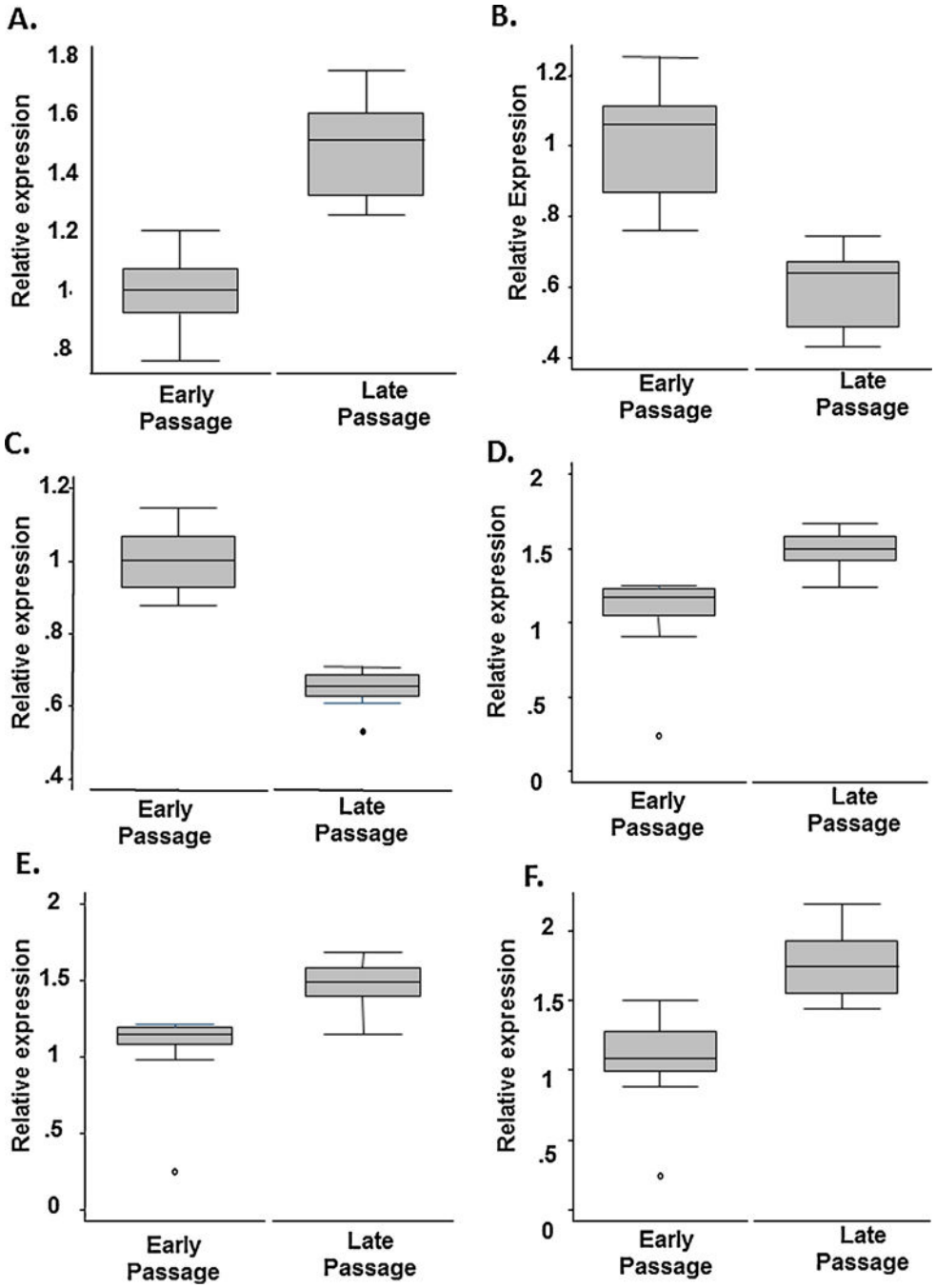

Fig. 4.

Box plots of the top 3 splicing factor gene expressions changes obtained by TaqMan low-density array (TLDA) in normal Human Dermal Fibroblasts (A–C) and Human Aortic Endothelial Cells (D–F). Gene expression levels are given relative to endogenous controls in young and old fibroblasts (passage 7 and passage 18 respectively, and in young and old endothelial cells (passage 7 and passage 13 respectively). On the *Y*-axis are gene expression levels relative to endogenous controls by cell passage (*X*-axis). Changes in the expression of

PNISR (**A**), *HNRNPA1* (**B**), *HNRNPA2B1* (**C**), *HNRNPUL2* (**D**), *HNRNPK* (**E**) and *HNRNPM* (**F**) transcripts with increasing passage are given.

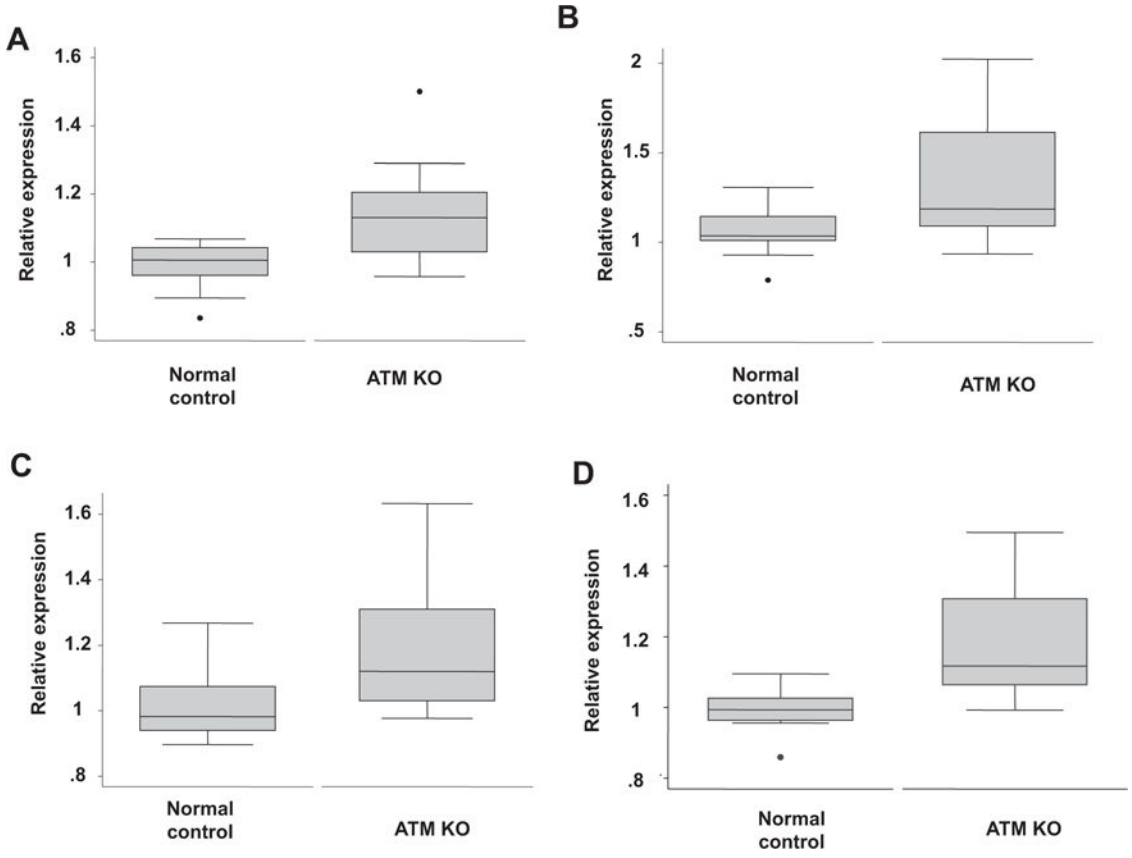

Fig. 5.

The top 4 splicing factor gene expression changes obtained by TaqMan low-density array (TLDA) in normal Human Dermal Fibroblasts treated with ATM siRNA. A = SRSF1, B = HNRNPA1, C = TRA2B and D = SRSF3 are given in this figure. Gene expression levels are given relative to endogenous controls in control fibroblasts at passage 9. These results represent the top 4 splicing factors (*SRSF1, HNRNPA1, TRA2B* and *SRSF3*) demonstrating increased expression in response to targeted knockdown of the *ATM* gene.

Table 1

The association of splicing factor gene expression with advancing age in the InCHIANTI and SAFHS populations ($n = 695$ and $n = 1238$ respectively) is given. Associations with age are calculated by logistic regression with adjustment for confounding factors. The gene and probe identities are given; the significant p value, coefficient and the false discovery Rate (FDR) adjusted q-value are given. Genes demonstrating an association with age at the level of $q = 0.05$ are given in bold, underlined font in both cases. Genes significantly associated with age in both datasets are indicated with a star.

InCHIANTI					SAFHS				
Gene ID	**Probed**	***p*-Value**	**Coefficient**	***q*-Value**	**Gene ID**	**Probed**	***p*-Value**	**Coefficient**	***q*-Value**
HNRNPA0	ilmn_1753279	<0.001	−0.002	<0.001	**HNRNPDL**	gi_14110406a	<0.001	−0.010	<0.001
SRSF17A	ilmn_2117716	<0.001	−0.002	<0.001	**LSM5***	gi_6912487s	<0.001	−0.010	<0.001
SRSFS6*	ilmn_1697469	<0.001	−0.003	<0.001	**LSM7**	gi_7706422s	<0.001	−0.008	<0.001
IMP3	ilmn_1733696	<0.001	−0.002	<0.001	**SRSF1***	gi_31543618s	<0.001	−0.007	<0.001
SRSF18	ilmn_2161357	<0.001	−0.001	<0.001	**LSM2***	gi_34013512s	<0.001	−0.006	0.001
HNRNPA1	ilmn_1661346	<0.001	−0.002	<0.001	**HNRNPH3***	gi_14141158a	0.002	−0.005	0.006
HNRNPD*	ilmn_2321451	<0.001	−0.003	0.001	**SRSF14***	gi_38490530s	0.002	0.005	0.006
SRSF1*	ilmn_1795341	<0.001	−0.002	0.001	**SRSF6***	gi_38158029s	0.002	−0.005	0.006
HSSG1	ilmn_1778836	<0.001	−0.001	0.001	**LSM3**	gi_7657314s	0.004	−0.005	0.009
SRSF10	ilmn_1742798	<0.001	−0.002	0.002	**HNRNPR**	gi_14141188s	0.005	−0.005	0.009
HNRNPM	ilmn_1805371	<0.001	−0.001	0.003	**SF3B1***	gi_6912653s	0.005	−0.005	0.010
HNRNPH3*	ilmn_1654920	0.001	−0.001	0.004	**RALY**	gi_21396479a	0.007	0.005	0.013
LSM5*	ilmn_1737947	0.001	−0.001	0.005	**HNRNPD***	gi_14110413a	0.010	−0.004	0.016
LSM2*	ilmn_2070300	0.001	−0.001	0.005	**HNRNPAB***	gi_14110401a	0.019	0.004	0.029
HNRNPUL2	ilmn_1810327	0.002	−0.001	0.007	**HNRNPH1**	gi_5031752s	0.032	−0.004	0.045
LSM14A	ilmn_2079803	0.003	−0.001	0.008	SRSF8	gi_23111063a	0.039	−0.003	0.052
SRSF14*	ilmn_1689007	0.003	0.000	0.008	SRSF12	gi_21040254s	0.043	−0.003	0.056
HNRNPK	ilmn_2378048	0.003	−0.002	0.008	SRSF3	gi_31377552s	0.048	−0.003	0.060
SRSF2	ilmn_1696407	0.004	−0.002	0.011	SF3A3	gi_5803166s	0.057	−0.003	0.067
SRSF5	ilmn_2378868	0.006	−0.002	0.015	LSM8	gi_21314665s	0.059	−0.003	0.069
SRSF3	ilmn_1723212	0.006	0.000	0.015	SRSF10	gi_4759097s	0.087	−0.003	0.094
SRSF11	ilmn_1657790	0.008	0.000	0.018	HNRNPA2B1	gi_14043073a	0.103	−0.003	0.107
HNRNPA2B1	ilmn_1886493	0.008	−0.001	0.019	SRSF16	gi_5902129s	0.108	0.003	0.111
U2AF2	ilmn_1768930	0.010	−0.001	0.023	SRSF2	gi_4506898s	0.138	0.003	0.133

InCHIANTI					SAFHS				
Gene ID	**Probed**	***p*-Value**	**Coefficient**	***q*-Value**	**Gene ID**	**Probed**	***p*-Value**	**Coefficient**	***q*-Value**
SF3B1*	ilmn_1706075	0.014	−0.002	0.029	HNRNPK	gi_14165436a	0.174	−0.002	0.155
HNRNPAB*	ilmn_1651262	0.022	−0.001	0.041	LSM10	gi_14249631s	0.178	0.002	0.158
SRSF9	ilmn_1760683	0.023	−0.001	0.043	HNRPL	gi_4557644s	0.181	0.002	0.160
SRSF8	ilmn_1692575	0.031	0.000	0.053	HNRNPA1	gi_4504444a	0.192	−0.002	0.165
SF3A1	ilmn_1697286	0.036	0.000	0.059	LSM6	gi_5901997s	0.218	−0.002	0.179
SRSF2IP	ilmn_1830806	0.036	0.000	0.059	SRSF2IP	gi_4759171s	0.236	−0.002	0.187
HNRNPH2	ilmn_1781764	0.037	0.000	0.060	HNRNPUL1	gi_21536323a	0.264	0.002	0.200
HNRNPF	ilmn_1668179	0.040	0.000	0.062	HNRNPM	gi_14141151i	0.305	0.002	0.218
SNRNP27	ilmn_2069945	0.041	0.000	0.063	SRSF4	gi_34147660s	0.306	0.002	0.219
SRSF15	ilmn_1659874	0.048	0.000	0.069	HNRNPC	gi_14110430a	0.329	−0.002	0.228
SRSF12	ilmn_2373266	0.054	0.000	0.074	IMP4	gi_15529981s	0.332	−0.002	0.229
LSMD1	ilmn_1691131	0.064	0.000	0.084	SF3B3	gi_40254848s	0.335	−0.002	0.230
SNRNP48	ilmn_1754304	0.078	0.000	0.098	SF3A1	gi_34147572s	0.342	0.002	0.234
SNRNP25	ilmn_1801118	0.087	0.000	0.107	SF3A2	gi_32189413s	0.425	−0.001	0.275
HNRNPC	ilmn_2334587	0.089	0.000	0.109	U2AF1	gi_5803206s	0.439	0.001	0.281
HNRNPA3	ilmn_1703369	0.103	0.000	0.122	HNRNPM	gi_14141153a	0.457	0.001	0.289
LSM12	ilmn_2092693	0.108	0.000	0.126	HNRNPAB	gi_14110403i	0.557	0.001	0.332
LSM6	ilmn_1675462	0.127	0.000	0.140	LSM1	gi_7657312s	0.566	0.001	0.335
HNRNPH1	ilmn_2101920	0.133	0.000	0.145	SF3B4	gi_23111059s	0.701	0.001	0.384
SNRNP35	ilmn_2395285	0.135	0.000	0.146	HNRNPU	gi_14141160a	0.767	−0.001	0.406
SF3B4	ilmn_1722648	0.137	−0.001	0.147	HNRNPA3	gi_34740328s	0.817	0.000	0.422
SF3B3	ilmn_1803110	0.152	0.000	0.158	SRSF11	gi_23111060s	0.843	0.000	0.429
HNRNPU	ilmn_2370135	0.172	0.001	0.174	U2AF2	gi_6005925s	0.848	0.000	0.430
SF3A3	ilmn_1705151	0.204	−0.001	0.199	HNRPF	gi_14141150s	0.850	0.000	0.431
SNRNP200	ilmn_1705928	0.225	0.000	0.213	SRSF15	gi_40789228s	0.858	0.000	0.434
SNRNP70	ilmn_1732053	0.229	−0.001	0.217	SRSF5	gi_40254839s	0.890	0.000	0.442
U2AF1L4	ilmn_1779177	0.271	0.000	0.244	HNRNPA0	gi_14110425s	0.940	0.000	0.456
LSM4	ilmn_1788099	0.278	0.000	0.249	SF3B5	gi_42740890s	0.943	0.000	0.457
SRSF16	ilmn_2146566	0.282	0.000	0.251	SRSF9	gi_38016912s	0.962	0.000	0.462
SRSF4	ilmn_2175075	0.300	−0.001	0.262	LSM4	gi_33620778s	0.986	0.000	0.468

InCHIANTI					SAFHS				
Gene ID	**Probed**	***p*-Value**	**Coefficient**	***q*-Value**	**Gene ID**	**Probed**	***p*-Value**	**Coefficient**	***q*-Value**
RRP8	ilmn_2066667	0.301	0.000	0.263	SF3B2	gi_37541034s	0.990	0.000	0.469
SF3B14	ilmn_2182120	0.348	0.000	0.291					
HNRNPUL1	ilmn_2395728	0.361	0.000	0.299					
LSM7	ilmn_1678165	0.414	0.000	0.329					
U2AF1	ilmn_1772113	0.433	0.000	0.339					
LSM3	ilmn_2229242	0.535	0.000	0.388					
LSM10	ilmn_1751803	0.553	0.000	0.395					
RRP9	ilmn_1689972	0.556	0.000	0.397					
LSM1	ilmn_2218450	0.571	0.000	0.403					
LSM8	ilmn_1805590	0.584	0.000	0.409					
IMP4	ilmn_2156982	0.594	0.000	0.412					
HNRNPL	ilmn_2389582	0.669	0.000	0.442					
SNRNP40	ilmn_1799814	0.708	0.000	0.456					
SF3A2	ilmn_1754220	0.710	0.000	0.456					
HNRNPR	ilmn_2175894	0.752	0.000	0.471					
SF3B2	ilmn_1775939	0.818	0.000	0.492					
SF3B5	ilmn_1689389	0.991	0.000	0.539					

Table 2

Molecular markers of cell senescence in young and old fibroblasts and endothelial cells

The change in the expression of age-related genes VEGFA and CDKN2A in fibroblasts and endothelial cells is given for “young” (passage = 7) and “old” fibroblasts (passage = 18) and endothelial cells (passage = 13). Differences in transcript expression levels were assessed by Mann–Whitney *U* analysis. The staining percentage of β-galactosidase marker of senescence is given for each passage. PD = population doubling and PDT = population doubling time in hours.

	Fibroblasts		**Endothelial cells**	
	p7	**p18**	**p7**	**p13**
β-gal (%)	4%	58%	7%	68%
	z	**p**	**z**	**p**
VEGFA	3.703	0.0002	4.157	0.0002
CDKN2A	−2.842	0.0045	−2.242	<0.001
PD	23	45	21	33
PDT (hours)	72.041	120.06	36.02	240.139

Table 3

Expression of core splicing factor and splicing regulator transcripts in cells senesced in vitro

Gene expression measurements were taken for twenty core splicing factor or splicing regulator genes in two cell populations (primary endothelial cells and fibroblasts). Gene expression between old and young passage cells (p7 and p13 in endothelial cells; p7 and p18 in fibroblasts) was assessed. Effect size and direction is given by *z* scores (standard deviation from the mean), *p*-values were determined by Mann–Whitney *U* analysis.

	Fibroblasts		Endothelial cells	
Gene	***z***	***p*-value**	***z***	***p*-value**
AKAP17A	3.926	0.0001	−2.771	0.0056
HNRNPA0	3.349	0.0008	−0.924	0.3556
HNRNPA1	4.157	<0.0001	−2.194	0.0282
HNRNPA2B1	4.157	<0.0001	−1.097	0.2727
HNRNPD	4.041	0.0001	0.000	1.0000
HNRNPH3	3.522	0.0004	0.173	0.8625
HNRNPK	3.753	0.0002	−3.811	0.0001
HNRNPM	2.771	0.0056	−4.041	0.0001
HNRNPUL2	−3.060	0.0022	−4.041	0.0001
IMP3	3.291	0.0010	−3.753	0.0002
LSM14A	3.002	0.0027	−3.464	0.0005
LSM2	2.078	0.0377	1.848	0.0647
PNISR	−4.157	<0.0001	2.714	0.0067
SF3B1	−2.078	0.0377	1.559	0.1190
SRSF1	3.522	0.0004	−0.981	0.3263
SRSF2	3.984	0.0001	0.231	0.8174
SRSF3	3.522	0.0004	1.097	0.2727
SRSF6	3.637	0.0003	−1.328	0.1842
SRSF7	4.157	<0.0001	3.291	0.0010
TRA2B	4.041	0.0001	−0.058	0.9540

Table 4

gene expression of alternatively expressed isoform ratios of age-related genes

Gene expression measurements were taken for genes known to be associated with ageing in primary human fibroblasts and primary endothelial cells. Their association with cellular age is calculated by Mann–Whitney *U* statistical analysis between expression in old and young passage cells (p7 and p13 in endothelial cells; p7 and p18 in fibroblasts).

GENE	PROBE ID	Isoform(s)	Fibroblasts	Endothelial cells				
			z	Prob > *z*	*z*	Prob > *z*	Differences in isoforms	Size of exon (bp)
CDKN2A	Hs00923894_m1	NM_000077.4	−4.099	<0.0001	−1.097	0.2727		exon 1 – 456
		NM_058195.3					Alternative exon 1	exon 1 – 353
		NM_058197.4					Alternative exon 1	exon 1 – 424
	Hs99999189_m1	NM_058195.3	1.559	0.119	0.289	0.7728	Alternative exon 1	exon 1 – 353
	Ratio		−3.811	0.0001	−1.848	0.0647		
VCAN	hs01007937_m1	NM_001164097.1	−3.984	0.0001	NA	NA		exon 7 – 5261
	hs01007943_m1	NM_001164098.1	−4.157	<0.0001	NA	NA	Alternative exon 7	exon 7 – 2960
	Ratio		4.157	<0.0001	NA	NA		
EFNA1	hs00358887_m1	NM_004428.2	NA	NA	4.157	<0.0001	exon 3 inclusion	exon 3 – 65
	hs01020895_m1	NM_182685.1	NA	NA	3.926	0.0001		
	Ratio		NA	NA	−1.443	0.1489		
VEGFA	Hs00903129	NM_003376.5	4.157	<0.0001	3.703	0.0002	exon 6 included	exon 6 –71
		NM_001171624.1						
	Hs00900057	NM_001171629.1	3.098	0.0019	3.34	0.0008	Alternative exon 6 excluded	
		NM_001171627.1					Alternative exon 6 excluded	
		NM_001171626.1					Alternative exon 6 excluded	
		NM_001033756.2					Alternative exon 6 excluded	
		NM_001025369.2					Alternative exon 6 excluded	
		NM_001025368.2					Alternative exon 6 excluded	
	Ratio		−1.903	0.057	−3.703	0.0002		

Table 5

Expression of core splicing and splicing regulator transcripts in siRNA-mediated transcript knockdown of the ATM gene

Gene expression measurements for twenty transcripts in siRNA-mediated transcript knockdown of the ATM gene. Gene expression is relative to endogenous controls and relative to normal controls. Mann–Whitney *U* analysis was used to assess gene expression.

Gene	*z*-value	*p*-value
AKAP17A	−1.039	0.2987
HNRNPA0	−1.212	0.2253
HNRNPA2B1	−0.981	0.3263
HNRNPD	−2.252	0.0243
HNRNPH3	−0.866	0.3865
HNRNPK	−1.617	0.106
HNRNPM	−1.386	0.1659
HNRNPUL2	0.058	0.954
HNRNPA1	−2.483	0.013
LSM14A	−2.078	0.0377
LSM2	−1.617	0.106
PNISR	−0.981	0.3263
SF3B1	−1.097	0.2727
SRSF1	−2.598	0.0094
SRSF2	−2.021	0.0433
SRSF3	−3.349	<0.001
SRSF6	−0.404	0.6861
SRSF7	−2.252	0.0243
TRA2B	−2.483	0.013
IMP3	−0.693	0.4884