Mechanisms of Ageing and Development 134 (2013) 356–366

Contents lists available at SciVerse ScienceDirect

Mechanisms of Ageing and Development

journal homepage: www.elsevier.com/locate/mechagedev

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

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ARTICLE INFO

Article history:
Received 31 January 2013
Received in revised form 16 May 2013
Accepted 25 May 2013
Available online 6 June 2013

Keywords:
Ageing
Splicing
SRSF
hnRNP
Human

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.

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

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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http://dx.doi.org/10.1016/j.mad.2013.05.006

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 $q < 0.05$ 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 μg/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