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Comparison of senescence-associated miRNAs in primary skin and lung fibroblasts

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Abstract

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MicroRNAs are non-coding RNAs with roles in many cellular processes. Tissue-specific miRNA profiles associated with senescence have been described for several cell and tissue types. We aimed to characterise miRNAs involved in core, rather than tissue-specific, senescence pathways by assessment of common miRNA expression differences in two different cell types, with follow-up of predicted targets in human peripheral blood. MicroRNAs were profiled in early and late passage primary lung and skin fibroblasts to identify commonly-deregulated miRNAs. Expression changes of their bioinformatically-predicted mRNA targets were then assessed in both cell types and in human peripheral blood from elderly participants in the InCHIANTI study. 57/178 and 26/492 microRNAs were altered in late passage skin and lung cells respectively. Three miRNAs (miR-92a, miR-15b and miR-125a-3p) were altered in both tissues. 14 mRNA targets of the common miRNAs were expressed in lung and skin fibroblasts, of which two demonstrated up-regulation in late passage skin and lung cells (*LYST*; $p = 0.02$ [skin] and 0.02 [lung] *INMT*; $p = 0.03$ [skin] and 0.04 [lung]). *ZMPSTE24* and *LHFPL2* demonstrated altered expression in late passage skin cells only ($p = 0.01$ and 0.05 respectively). *LHFPL2* was also positively correlated with age in peripheral blood (p value = 6.6×10^{-5}). We find that the majority of senescence-associated miRNAs demonstrate tissue-specific effects. However, miRNAs showing common effects across tissue types may represent those associated with core, rather than tissue-specific senescence processes.

Keywords

Cellular senescence; microRNA; Tissue specificity; Gene expression

Introduction

Aging is a complex process characterised by progressive deterioration in cellular, tissue and organismal function. As such, aging in man is associated with almost all common chronic diseases (Butler et al. 2008). However, the decline in function is not uniform, with people aging at different rates. This heterogeneity of human aging and susceptibility to age-related disease are poorly understood and the molecular mechanisms that underlie these differences remain to be explained.

A proportion of the heterogeneity could arise from inter-individual variability in the fine-tuning of gene expression. Studies in multiple tissues from several species have identified genes where transcript expression or mRNA processing are associated with aging (Glass and McClusky 1987; Harries et al. 2011; Holly et al. 2013; Weindruch et al. 2002). Gene expression can be regulated at many points. At transcription, expression can be moderated by the presence within the cell of appropriate transcription factors, or by epigenetic changes such as DNA methylation or histone modifications which determine whether the heterochromatin is in an active state (Jaenisch and Bird 2003). Secondly, primary mRNA transcripts can undergo alternative mRNA splicing, which enables one gene to code for many proteins by moderating exon inclusion or exclusion (Breitbart et al. 1987). Finally, post-transcriptional regulation of mRNA stability or translation potential by microRNAs, or by RNA binding proteins can occur (Filipowicz et al. 2008).

MicroRNAs (miRNAs) are short non-coding RNAs around 22 nucleotides in length and represent crucial players in many physiological processes, including development, proliferation and many disease phenotypes (Soifer et al. 2007). MicroRNAs act on their mRNA targets by acting as guides to direct RNA-induced silencing complexes (RISC) to the target mRNA for degradation or translational repression (Gregory et al. 2005). Recognition of the target mRNA is through sequence complementarity of the mRNA to the ‘seed region’, the first 6–8 nucleotides of the microRNA. This complementarity allows binding to 3′ regions of the target mRNA transcripts and repressing their translation or targeting for degradation (Bartel 2009). There are currently over 1800 identified mature human microRNA sequences (Kozomara and Griffiths-Jones 2011) and each microRNA can target hundreds of mRNAs (Lim et al. 2005); many transcripts have multiple microRNA binding sites in their 3′ untranslated region, which may act antagonistically or be complementary to one another. MicroRNAs regulate the expression of genes in most pathways and biological processes (Stefani and Slack 2008) and have the potential to regulate complex networks such as those found during cellular aging (Inukai and Slack 2013).

In this study, we were interested in identifying miRNAs demonstrating common differences in expression in early and late passage cells of different types, as these may represent those important in the core senescence processes, rather than tissue-specific aspects. We therefore examined the microRNA expression changes present in late passage primary human skin and lung fibroblasts, compared with early passage controls of each cell type. We then assessed the fate of bioinformatically-predicted mRNA targets of common miRNAs in each tissue type, and in peripheral blood from a cross-sectional population study in order to identify potential biomarkers of senescence.

Materials and methods

miRNA and mRNA extraction and reverse transcription from early and late passage skin and lung fibroblasts

Tissue culture methods and protocols for in vitro cell senescence in skin fibroblasts and lung MRC5 fibroblasts have been previously described in (Holly et al. 2013) and (Passos et al. 2007) respectively.

MicroRNAs were extracted from ‘early’ (passage = 7, population doublings = 23) and ‘late’ passage skin fibroblasts (passage = 18, population doublings = 45) using the ‘mirVana™ miRNA Isolation Kit’ (Life Technologies, Foster City, USA) following the manufacturer’s instructions. Cellular senescence was confirmed by β -galactosidase staining with molecular assessment of senescence as previously described (Holly et al. 2013). Total RNA (350 ng) extracted using the mirVana system was reverse transcribed in 7.5 μ l reactions using the Megaplex™ RT Primers, Human pool A v2.1 and the TaqMan® MicroRNA reverse Transcription Kit (Life Technologies, Foster City, USA). Cycling conditions for the reverse transcription of the ABI 7900HT platform (Life Technologies, Foster City, USA) were 40 cycles 16 °C for 2 min, 42 °C for 1 min and 50 °C for 1 s followed by 85 °C for 5 min.

MRC-5 human embryonic lung fibroblasts (obtained from ECACC) were grown in Dulbecco’s Modified Eagle’s Medium (Sigma, UK) supplemented with 10 % fetal calf

serum, 2 mM L-glutamine, 1× penicillin/streptomycin (Sigma, UK). Fibroblasts were grown in a humidified atmosphere of 5 % CO₂ and 95 % air at ambient oxygen partial pressure at 37 °C on 75 cm² flasks (Corning Incorporated, USA), in 15 ml of medium. Cells were split at 90 % confluence into identical fresh medium. Cells were harvested by washing twice in phosphate buffered saline (PBS; Sigma, UK). MicroRNAs were extracted from 'early' passage (population doublings = 28.8) and 'late' passage MRC5 fibroblasts (population doublings = 43.6) using the 'mirVana[™] miRNA Isolation Kit' (Life Technologies, Foster City, USA) following the manufacturer's instructions. Total RNA extraction was carried out using the RNeasy kit (Qiagen) and cDNA synthesis was carried out using Superscript Vilo cDNA kit (Life Technologies) according to manufacturer's instructions.

MicroRNA expression profiling from 'Early' and 'Late' passage skin fibroblasts

Quantitative RT-PCR reactions for human skin fibroblast miRNAs were performed on the ABI 7900HT platform using TaqMan[®] Array Human MicroRNA A Cards v2.0 (Life Technologies, Foster City, USA) containing 384 TaqMan[®] MicroRNA Assays. Reaction mix contained 6 µl of Megaplex[™] RT product, 450 µl TaqMan[®] 2× Universal PCR Master Mix, No AmpErase[®] UNG and 444 µl nuclease-free water. 100 µl was dispensed in 8 chambers of each TLDA card and centrifuged twice for 1 min at 1000 rpm and sealed. Cycling conditions for the TLDA qRT-PCR reaction 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. Comparative Ct values were calculated to determine the expression of each microRNA transcript (Pfaffl 2001). Assessment of expression used a global normalisation strategy (Mestdagh et al. 2009), following removal of outliers with crossing points of <10 and >38. Statistical analyses were carried out using STATA v16.0 (StataCorp LP, Texas, USA). *t* test analysis was used to test any statistical significance of microRNA expression between the early and late passage cells. Data were Benjamini-Hochberg corrected to account for multiple testing, based on 178 miRNAs expressed in this tissue, with α set at $q = 0.05$.

MicroRNA and mRNA expression profiling in early and late passage lung Fibroblasts

MicroRNAs in primary lung fibroblasts were profiled by array technology using the ThermoScientific miRNA profiling system in early (proliferating) and late passage (senescent) cells. Relative intensity data was subjected to statistical filtering, retaining miRNA probes with p value ≤ 0.01 . This resulted in 492 miRNA probes passing statistical filters. Data was inter-array scaled and transformed to log₂. This annotated, filtered, scaled and log₂-transformed data set was used for subsequent analysis. Differential expression between samples was calculated using empirical Bayes statistics on the normalised expression data after the data was fitted to a linear model using the LIMMA Bioconductor package (Smyth 2004).

Differential mRNA expression on human lung fibroblasts was undertaken using an Agilent whole genome human 4 × 44 K Gene expression array. The relative intensity data was normalised using the RMA algorithm (Robust Microarray Average) using the Bioconductor package Affy (Gautier et al. 2004). The normalised data was then fitted to a linear model using the LIMMA package (Smyth 2004) and changes in gene expression calculated using empirical Bayes statistics.

Human peripheral blood transcriptional analysis

We analysed microarray data from participants at the 9-year follow-up of the InCHIANTI study, which has been previously described (Ferrucci et al. 2000; Harries et al. 2012a, b; Holly et al. 2013). 695 subjects aged between 30 and 104 years old with a mean age of 72.3 years (standard deviation 15.3 years). Full characteristics of the population used in this study are given in Harries et al. 2011). Whole transcriptome profiles were generated from using Illumina HT-12 microarray technology (Illumina, San Diego, California, USA) (Zeller et al. 2010). Array data can be accessed in the Gene Expression Omnibus database under accession number GSE48152. The InCHIANTI population is a well characterised, large cross sectional population study which has been extensively used for the study of human aging traits (Ferrucci et al. 2000). Ethical approval was granted by the Istituto Nazionale Riposo e Cura Anziani institutional review board.

Identification of target genes of the most senescence-associated microRNAs

We used the miRNA target predictor web interface MirWalk (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/>) to identify putative targets of the three microRNAs demonstrating significantly altered expression in a consistent direction in early and late passage lung and skin fibroblasts (Dweep et al. 2011). We searched within the 3' UTR binding sites for minimum seed length of seven for the longest transcripts of the human genes, in five comparative prediction programs (miRanda, miRDB, miRWalk, RNA22 and Targetscan) (Dweep et al. 2011).

Assessment of expression status of bioinformatically-predicted target genes in skin and lung fibroblasts

RNA from early and late passage primary lung and skin fibroblasts were used for demonstrating expression changes in predicted target genes to the three common miRNAs showing common expression differences in both fibroblast types, using qRT-PCR. SYBR green qPCR amplification was performed using SensiFAST™ SYBR® No-ROX Kit (Bioline, UK). qPCR reactions were carried out in a total reaction volume of 20 µl, containing 2× SensiFAST™ SYBR® No-ROX Mix, 8 µM each of forward and reverse primers and 1 µl template cDNA. Reactions were carried out using a Rotor-Gene 3000 system (Qiagen, Germany) using following parameters: polymerase activation for 2 min at 95 °C, and 40 cycles of denaturation for 5 s at 95 °C, annealing for 10 s at 60 °C and extension for 20 s at 72 °C. A melt curve was carried out from 55 to 95 °C to determine product purity. Rotor-Gene 6000 series 1.7 software was used for analysing and quantifying the data. A threshold value of 0.03097 was used for determination of all Ct values. qRT-PCRs were performed in technical duplicates or triplicates and biological triplicates. Results of the technical duplicates were averaged. Ct-values of our genes of interest were normalised using Ct-values of GAPDH. Error-bars were calculated from the standard deviation of the biological triplicates of these normalized Ct-values. The rules for propagation of uncertainty were followed to calculate the standard deviations on the final fold change values. The normalized Ct-values were averaged, after which the log2-fold-change and the fold change were calculated using the Livak method.

Assessment of expression status of bioinformatically-predicted target genes in human peripheral blood from an aging population

We also assessed the relationship between gene expression and age for the 19 genes predicted to be targets of the two common miRNAs in microarray data from peripheral blood samples from an aging population (the InCHIANTI study) by multivariate linear regression analysis. Regression analysis was carried out using the R statistical package v3 (New Jersey, USA). Models were adjusted for confounding factors including batch effects, sex, waist circumference (continuous trait), smoking status in lifetime pack years smoked (in four categories: none, <20, 20–39, 40+ years) education level in five categories (none, elementary, secondary, high school, and university), study site and white cell subtype count (neutrophil, lymphocyte, monocyte and eosinophil percentages).

Cubic spline analysis to assess linearity of association

Penalized cubic regression spline smooths (splines) were used in generalized additive models (GAM) using package “mgcv” (v1.8–3) (Wood 2011) for the R statistical language (v3.1.1) (R-Core-team 2014) to assess the linearity of gene expression associations with age. The models were formed in the same way as described elsewhere in the manuscript: gene expression values were the dependent variable, and age, sex, waist, education, smoking, study site, batches and cell counts were included as independent variables. Age was the spline term, with five knots specified for each analysis. The p value reported is from the GAM model relating to the association between age and the gene expression values, however in contrast to linear regression methods there is no assumption of linearity.

Results

57 miRNAs are associated with replicative senescence in primary human skin fibroblasts

We assessed the profile of microRNA expression in early and late passage primary skin fibroblasts. 178 out of 380 microRNAs tested were expressed in early or late passage fibroblasts. Of these, 57 were significantly associated with senescence following Benjamini Hoch-berg adjustment for multiple testing. All microRNA transcripts had lower expression in late passage fibroblasts except hsa-miR-138, which had threefold higher expression in the late passage skin fibroblasts (Table 1). Among the most down-regulated microRNAs in senescence include miR-27b, miR-455, miR-337-5p, miR-135b and miR-579, ($p = 7.6 \times 10^{-8}$, 1.4×10^{-7} , 2.0×10^{-7} , 2.4×10^{-7} and 2.0×10^{-6} respectively).

26 miRNAs are associated with replicative senescence in primary human lung fibroblasts

We assessed the profile of microRNAs expressions in early and late passage primary lung fibroblasts. 492 microRNAs tested were expressed in primary lung fibroblasts. 26 of these were significantly associated with cellular senescence following empirical Bayes testing (Table 2). Eight miRNAs were down-regulated and 18 were up-regulated. The most down-regulated miRNAs in primary lung fibroblasts were hsa-miR-155 and hsa-miR-484 both of which showed a drop in expression by almost half. In contrast hsa-miR-127-3p ($p = 5.1 \times 10^{-4}$) and hsa-miR-768-3p ($p = 4.2 \times 10^{-4}$) showed the greatest level up-regulation with both more than doubling their expression during senescence.

Senescent cells of different lineages demonstrate tissue-specificity in miRNA profiles

380 miRNAs were tested by qRT-PCR in skin fibroblasts and 703 miRNAs were tested by microarray in the lung fibroblasts. 365 miRNAs were common to both arrays. 178/380 miRNAs were expressed in skin fibroblasts and 492/703 miRNAs were expressed in lung fibroblasts. 174 miRNAs were present and expressed in both lung and skin cells. Despite this considerable overlap, only 3 of 174 (1.7 %) commonly expressed miRNAs were associated with cellular senescence in both tissue types. The majority of miRNAs therefore demonstrated tissue-specificity in their expression in response to cellular senescence, with only three miRNAs, miR-92a, miR-125a-3p and miR-15b demonstrating consistent effects with senescence in both tissue types (Tables 1, 2).

miRNAs demonstrating similar expression differences in early and late passage lung and skin fibroblasts are predicted to target 19 mRNAs

We employed bioinformatics to identify putative targets of the three microRNAs associated with replicative senescence in both skin and lung fibroblasts. The three microRNAs in question, miR-92a and miR-15b, were predicted to regulate 19 mRNAs as determined by MirWalk prediction (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/>). Of these 19 genes, 14 were expressed in both skin and lung fibroblasts when assessed by qRT-PCR. Two mRNA targets, *LYST* and *INMT*, showed expression differences in lung fibroblasts and four in skin fibroblasts (Table 3; Fig. 1). The lysosomal trafficking regulator (*LYST*) gene, predicted to be targeted by miR-92a demonstrates 4.4× and 2.5× higher expression in late passage lung and skin fibroblasts respectively ($p = 0.02$ and 0.02), whereas the indolethylamine *N*-methyltransferase (*INMT*) gene, predicted to be regulated by miR-125a-3p, demonstrates 3.3× and 4.3× higher expression in late passage lung and skin cells respectively ($p = 0.02$ and 0.03). The lipoma HMGIC fusion partner-like 2 (*LHFPL2*) gene predicted to be regulated by miR-92a, and the zinc metalloproteinase STE24 (*ZMPSTE24*) gene, predicted to be targeted by miR-125a-3p, both demonstrate elevated expression in late passage skin fibroblasts only (1.8× higher expression; $p = 0.05$ and 1.6× higher expression; $p = 0.01$ for *LHFPL2* and *ZMPSTE24* respectively).

Some miRNA target transcripts also demonstrate age-associated expression differences in peripheral blood

11/19 mRNAs bioinformatically-predicted to be targeted by miR-92a or miR-15b are also expressed in peripheral blood. In a regression analysis of samples from a population based study of aging, the INCHIANTI study. Of these, *LHFPL2*, *LYST*, *ZMPSTE24* and *CCDC28A* demonstrated robust associations with age following correction for multiple testing using a p value threshold of 0.005. The majority of these associations were inverse correlations, in contrast to the skin and lung fibroblast data, although *LHFPL2* demonstrated consistency in direction of effect (Table 4). Cubic spline analysis demonstrated that whilst two of the genes (*LYST* and *ZMPSTE24*) demonstrated a linear association with age, two (*CCDC28A* and *LHFPL2*) did demonstrate some degree of non-linearity (supplementary Fig. 1). However, these associations remained statistically significant ($p = 0.01$ and 0.0003 respectively) and it should be noted that effects on linearity may be driven by reduced power in the younger samples since the majority of the sample were aged 75 or above.

Discussion

We have identified senescence-associated changes in the mRNA milieu in early and late passage primary cells of two lineages; skin fibroblasts and lung fibroblasts. 57 miRNAs demonstrated altered expression in late passage skin fibroblasts, and 20 miRNAs demonstrated differential expression in late passage lung fibroblasts. Senescence-associated miRNA profiles demonstrated considerable tissue specificity, but three miRNAs, miR-92a and miR-15b showed similar differences in both datasets. MicroRNAs associated with senescence in multiple cell and tissue types may represent those more involved in regulation of 'core' aging processes that occur in multiple cell types, rather than representing more tissue-specific aspects of senescence.

There has been accumulating evidence implicating microRNA regulation of aging genes in model organisms (Boehm and Slack 2005; Liu et al. 2012). MicroRNA studies in humans have indicated that the majority of microRNAs decrease in abundance with age (Noren Hooten et al. 2010), and our data are concordant with these observations in human skin fibroblasts, although the lung fibroblasts show more variability in this respect. In particular, miR-17, miR-19b, miR-20a and miR-106a demonstrate reduced expression with senescence in late passage skin fibroblasts, and have previously reported to demonstrate reduced expression in four additional aging models, including another study of skin fibroblasts and three in vivo animal models (Hackl et al. 2010).

Elderly organisms have been described to display several 'hallmarks' of aging, including genome damage, telomere attrition, deregulated nutrient sensing, altered epigenetics, disrupted proteostasis, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication (Lopez-Otin et al. 2013). Accordingly, several of the miRNAs demonstrating expression differences in early and late passage primary skin fibroblasts and primary lung fibroblasts have been reported to regulate genes involved in these pathways. Several members of the Let-7 family were found to be down-regulated in late passage skin fibroblasts; this miRNA family has been reported to be involved in cellular senescence and stem cell exhaustion, by virtue of their regulation of the *HGMA2* and *CDKN2A* transcripts (Nishino et al. 2008). Similarly, reduced expression of miR-24, present at reduced levels in late passage skin fibroblasts in our study, has previously been demonstrated to result in an increased expression of *CDKN2A*, which encodes p16ink4A and ARF, key markers of senescence in vivo and in vitro (Lal et al. 2008). Components of the telomere maintenance machinery are also known to be targeted by miR-138 and miR-155. The *TRF1* transcript, which encodes the telomeric repeat binding factor 1A, a critical component of the telomere assembly apparatus, is targeted by miR-155, which shows altered expression in late passage primary lung fibroblasts. Telomerase itself is regulated by miR-138, present at reduced levels in late passage primary skin fibroblasts. Components of the nutrient sensing and proteostatic pathways are also targeted by miRNAs showing altered expression in late passage skin fibroblasts; the *PTEN* gene, a key regulator of IGF1 and mTOR signalling is targeted by miR-19b, miR-17 miR-106a and miR-20a (Grillari et al. 2010).

The three miRNAs displaying consistent expression differences in late passage lung and skin cells, miR-92a and miR-15b were predicted by bioinformatics to target 19 mRNA transcripts. Of these, the *LYST* and *INMT* were present at elevated levels in both cell types. The *LYST* gene encodes the lysosomal trafficking regulator protein, which regulates movement of proteins within lysosomes. Mutations in this gene are associated with Chediak-Higashi syndrome, a lysosomal storage disorder characterised by pigmentation changes, susceptibility to infection and bleeding abnormalities. It is fatal in its accelerated stages (Roy et al. 2011). Mice with mutated *LYST* genes are prone to fibrosis during aging (Wang and Lyerla 2010) and Rab-LYST trafficking was also found to be altered in a mouse model of Alzheimer's disease (Soreghan et al. 2005). Dysregulated autophagy, a key marker of aging, is also a known outcome of lysosomal storage disorders (Papackova and Cahova 2014).

The three miRNAs displaying consistent expression differences in senescent lung and skin cells, miR-92a, miR-125a-3p and miR-15b were predicted by bioinformatics to target 19 mRNA transcripts. Of these, the *LYST* and *INMT* were present at elevated levels in both cell types. The *LYST* gene encodes the lysosomal trafficking regulator protein, which regulates movement of proteins within lysosomes. Mutations in this gene are associated with Chediak-Higashi syndrome, a lysosomal storage disorder characterised by pigmentation changes, susceptibility to infection and bleeding abnormalities. It is fatal in its accelerated stages (Roy et al. 2011). Mice with mutated *LYST* genes are prone to fibrosis during aging (Wang and Lyerla 2010) and Rab-LYST trafficking was also found to be altered in a mouse model of Alzheimer's Disease (Soreghan et al. 2005). Dysregulated autophagy, a key marker of aging, is also a known outcome of lysosomal storage disorders (Papackova and Cahova 2014). The *INMT* gene, encodes the indolethylamine *N*-methyltransferase protein which is involved in xenobiotic metabolism. Age-related changes to the expression of this gene could potentially alter the activation or deactivation of carcinogens and therapeutic drugs in the elderly. Two genes, the HMGIC fusion partner-like two (*LHFPL2*) gene and the zinc metallopeptidase STE24 (*ZMPSTE24*) gene demonstrated elevated expression only in senescent skin fibroblasts. *LHFPL2* encodes a tetraspan transmembrane protein, mutations in which result in deafness in mice (Longo-Guess et al. 2005). *LHFPL2* was also found to be linked to differentiation of embryonic stem cells (Brandenberger et al. 2004), and so could have a role in stem cell exhaustion. The *ZMPSTE24* or *FACE1* gene encodes a metallopeptidase protein involved in the post-translational cleavage of Prelamin A to mature Lamin A. Lamin A is an important aging gene, which moderates the nuclear lamina to allow expression of multiple genes. Mutations at this locus cause Hutchinson-Gifford Progeria Syndrome (HGPS), an accelerated aging syndrome (Ghosh and Zhou 2014). Mutations at *ZMPSTE24* have also been reported in HGPS (Denecke et al. 2006).

Three out of four of the mRNA targets of miRNAs deregulated by senescence in skin or lung fibroblasts were also associated with advancing age in peripheral blood from a cross-sectional population study of the aging, the InCHIANTI study. Of these three genes, only *LHFPL2* was shown to demonstrate a common direction of effect. Differences in direction of effect between in vitro and in vivo aging studies are not uncommon; indeed we have noted this phenomenon in previous studies (Harries et al. 2011; Holly et al. 2013). There are several potential explanations for this phenomenon. Firstly, comparisons of a single homogeneous tissue such as a cell line with a mixed heterogeneous tissue such as blood can

sometimes produce discrepant results. Blood is a complex mixture of different cell types and changes in gene expression can sometimes arise because of changes in the composition of the blood cell pool. Secondly, population studies can be subject to other internal and external environmental factors that may affect the expression of genes. Individuals within the population may have been exposed to confounding factors that the cells have not been exposed to. Finally, in living populations, expression changes with detrimental systemic outcomes may not be represented in our elderly samples because of survivor effects.

The strengths of this study are the use of two different primary cell types within an in vitro model of human aging, with both miRNA and mRNA available from the same cells. This model enables the changes we are seeing do not represent a manifestation of other cell types or environmental factors and are not subject to confounding factors as seen found in human in vivo studies, however the direction and causality are attributable only to this cell type. We noted that three out of four genes demonstrating expression differences in both skin and lung fibroblasts also demonstrated altered expression in peripheral blood from elderly humans in a cross-sectional population study. This may indicate that these genes may have more general importance in the aging process.

A limitation to our study is that in our in vitro model, most cells are undergoing senescence, whereas this is probably not the case a rapidly dividing cell population which is replenished from the stem cell pool. However, senescent cells are certainly present in vivo; in the aging tissues of primates there is a senescent cell accumulation in vivo of more than 15 % senescent cells, and this indicates in vitro senescence is a credible model of human aging (Herbig et al. 2006; Jeyapalan and Sedivy 2008). It must also be noted that given the limitations of in vitro models of senescence, which often do not employ large numbers of biological replicates, we may have identified only moderate to large effects, and other miRNA targets which show no expression differences in our study may prove to do so in larger, better powered studies of primary tissues. Another limitation lies in the use of different technologies to measure miRNA expression. Despite this, a significant number of miRNAs (365) were tested on both arrays. Of these, only 3/174 (1.7 %) miRNAs that were expressed in both tissue types demonstrated associations with senescence, leading us to conclude that the small degree of overlap in senescence-associated miRNAs arises from tissue specificity rather than differences in the portfolio of miRNAs tested on the different platforms.

We have demonstrated that senescence-associated miRNA profiles demonstrate significant tissue specificity, but that some miRNAs appear to demonstrate consistent differences across cell types. Some bioinformatically-predicted targets of miRNAs common to different cell types show consistent changes in senescence- or age-associated expression, but again, some tissue-specificity is apparent. Targets common to more than one tissue types include genes potentially important in aging such as *ZMPSTE24* and *LYST*. Identification of miRNAs and their targets that are conserved not only in cell models of aging, but in aging people themselves may provide mechanistic insight into the aging process and suggest potential biomarkers for aging processes in man.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Target gene expression in senescent lung and skin fibroblasts. The expression of bioinformatically-predicted target genes of miR-92a, miR-125a-3p and miR-15b in **a** early and late passage lung fibroblasts and **b** early and late passage skin fibroblasts are given. Expression levels in senescent cells are given by *light grey boxes* and expression levels in non-senescent by *dark grey boxes*. *Error bars* represent standard deviation of measurement. Statistically significant results are indicated by *stars*

Table 1

MicroRNAs with differential regulation in senescent skin fibroblasts

MicroRNA	Relative expression		p value	SE	BH q-value
	Early	Late			
miR-27b	1	1.42E-05	7.61E-08	0.40	1.11E-05
miR-455	1	5.72E-05	1.41E-07	0.26	1.11E-05
miR-337-5p	1	1.34E-04	2.01E-07	0.40	1.11E-05
miR-135b	1	1.12E-04	2.47E-07	0.26	1.11E-05
miR-579	1	1.13E-04	2.04E-06	0.47	6.77E-05
miR-487a	1	1.73E-05	2.27E-06	0.51	6.77E-05
miR-18a	1	4.75E-05	3.69E-06	0.51	9.44E-05
miR-199a	1	5.73E-05	4.47E-06	0.52	9.67E-05
miR-100	1	0.17	4.86E-06	0.16	9.67E-05
miR-671-3p	1	6.73E-05	1.44E-05	0.58	2.58E-04
let-7e	1	0.33	2.92E-05	0.14	4.21E-04
miR-145	1	0.07	2.94E-05	0.33	4.21E-04
miR-21	1	0.07	3.06E-05	0.21	4.21E-04
miR-99a	1	0.20	3.65E-05	0.19	4.43E-04
miR-31	1	0.20	3.71E-05	0.20	4.43E-04
miR-221	1	0.14	1.12E-04	0.22	1.25E-03
miR-17	1	0.24	2.11E-04	0.20	2.22E-03
miR-502-3p	1	3.81E-04	2.35E-04	0.77	2.34E-03
miR-125b	1	0.28	2.60E-04	0.24	2.45E-03
miR-19b	1	0.20	4.41E-04	0.32	3.82E-03
miR-26a	1	0.20	4.55E-04	0.32	3.82E-03
miR-127	1	0.20	4.7E-04	0.31	3.82E-03
miR-106a	1	0.28	5.9E-04	0.211	4.58E-03
miR-20a	1	0.14	6.47E-04	0.44	4.83E-03
miR-199a-3p	1	0.24	8.57E-04	0.32	6.13E-03
miR-365	1	0.33	9.74E-04	0.26	6.71E-03
miR-130a	1	0.07	1.12E-03	0.47	7.44E-03

MicroRNA	Relative expression		p value	SE	BH q-value
	Early	Late			
miR-92a	1	0.24	1.30E-03	0.35	8.28E-03
miR-517b	1	0.20	1.49E-03	0.41	8.70E-03
miR-125a-3p	1	0.06	1.49E-03	0.23	8.70E-03
miR-138	1	3.16	1.50E-03	0.23	8.70E-03
miR-19a	1	0.17	2.06E-03	0.49	0.01
miR-132	1	0.12	2.20E-03	0.48	0.01
miR-27a	1	0.05	3.17E-03	0.61	0.02
miR-222	1	0.34	3.32E-03	0.33	0.02
miR-374	1	0.34	3.66E-03	0.34	0.02
miR-152	1	0.40	4.29E-03	0.29	0.02
miR-885-5p	1	9.1E-04	5.40E-03	1.47	0.02
miR-15b	1	0.12	5.40E-03	0.49	0.02
let-7d	1	0.23	5.56E-03	0.50	0.02
miR-140	1	0.24	5.60E-03	0.49	0.02
miR-93	1	0.18	5.66E-03	0.54	0.02
miR-532	1	0.10	6.30E-03	0.61	0.03
miR-193b	1	0.47	6.56E-03	0.26	0.03
miR-29a	1	0.40	7.00E-03	0.31	0.03
miR-99b	1	0.17	7.00E-03	0.46	0.03
let-7 g	1	0.40	7.12E-03	0.32	0.03
miR-618	1	0.01	0.01	1.21	0.04
miR-204	1	0.28	0.01	0.51	0.04
let-7a	1	0.24	0.01	0.49	0.04
miR-376c	1	0.56	0.01	0.23	0.04
miR-26b	1	0.17	0.01	0.58	0.05
miR-181a	1	0.07	0.01	0.94	0.05
miR-214	1	0.34	0.01	0.35	0.05
miR-30b	1	0.33	0.01	0.37	0.05
miR-24	1	0.47	0.01	0.28	0.05
miR-342-3p	1	0.56	0.02	0.22	0.05

MicroRNA expression was assessed in 'early', and 'late', passage. The microRNA identity is given (SE) and Benjamini Hochberg q value (BH q value) is given

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Table 2

MicroRNAs with differential regulation in senescent lung fibroblasts

miRNA	Relative expression		p value	BH q value
	Early passage	Late passage		
hsa-miR-768-3p	1	3.37	8.54E-07	4.20E-04
hsa-miR-127-3p	1	2.24	2.07E-06	5.09E-04
hsa-miR-484	1	0.57	9.83E-06	1.61E-03
hsa-miR-432	1	1.66	1.93E-05	2.25E-03
hsa-miR-92a	1	0.58	2.29E-05	2.25E-03
hsa-miR-493*	1	1.65	2.90E-05	2.38E-03
hsa-miR-487b	1	1.60	5.00E-05	3.52E-03
hsa-miR-152	1	1.67	6.27E-05	3.85E-03
hsa-miR-363*	1	0.66	1.20E-04	6.68E-03
hsa-miR-15b	1	0.61	1.70E-04	8.39E-03
hsa-miR-409-3p	1	1.561	2.37E-04	9.75E-03
hsa-miR-155	1	0.50	2.24E-04	9.75E-03
hsa-miR-663	1	1.58	2.66E-04	0.01
hsa-miR-151-5p	1	1.47	3.92E-04	0.01
hsa-let-7a	1	1.82	6.14E-04	0.02
hsa-miR-494	1	0.70	6.41E-04	0.02
hsa-let-7 g	1	1.63	7.96E-04	0.02
hsa-let-7c	1	1.61	9.99E-04	0.03
hsa-miR-26b	1	1.40	1.155E-03	0.03
hsa-miR-125a-3p	1	0.71	1.35E-03	0.03
hsa-miR-342-3p	1	1.40	1.62E-03	0.04
hsa-miR-98	1	1.52	1.79E-03	0.04
hsa-miR-654-3p	1	1.39	1.84E-03	0.04
hsa-miR-10b	1	0.73	2.08E-03	0.04
hsa-miR-22	1	1.86	2.27E-03	0.04
hsa-miR-134	1	1.43	2.24E-03	0.04

MicroRNA expression was assessed in 'early' and 'late' passage. The microRNA identity is given, along with the relative expression in the 'early' and 'late' passage lung fibroblasts, the p values, and Benjamini Hochberg q values (BH q values) are given. The asterisks are part of the miRNA name and indicate that the miRNA is a 'star' sequence (i.e. arising from the antisense strand).

Table 3

Quantification of candidate mRNAs targeted by miRNAs associated with senescence in both skin and lung fibroblasts in senescent skin and lung fibroblasts

miRNA target gene	miRNA	RQ non-senescent	RQ senescent	p value
Lung fibroblasts				
<i>ADAMTS5</i>	miR-15b	0.09 (0.04)	0.59 (0.29)	0.09
<i>ADCY3</i>	miR-92a	1.93 (1.73)	4.76 (1.69)	0.11
<i>CCDC28A</i>	miR-15b	1.27 (0.56)	4.94 (2.27)	0.1
<i>FAM134C</i>	miR-15b	1.05 (0.44)	3.3 (0.96)	0.15
<i>FRY</i>	miR-92a	0.85 (0.52)	1.62 (0.91)	0.29
<i>INMT</i>	miR-125a-3p	1.01 (0.22)	3.33 (0.86)	0.04
<i>IVNS1ABP</i>	miR-125a-3p	0.99 (0.26)	4.33 (4.5)	0.33
<i>LHFPL2</i>	miR-92a	0.36 (0.38)	0.46 (0.77)	0.85
<i>LYST</i>	miR-92a	1.01 (0.35)	4.45 (1.06)	0.02
<i>SLC36A1</i>	miR-15b	1.06 (0.28)	2.26 (1.39)	0.43
<i>TCFL5</i>	miR-92a	0.66 (0.48)	1.63 (1.06)	0.4
<i>TEX261</i>	miR-125a-3p	0.97 (0.40)	2.67 (1.45)	0.17
<i>ZFP36L1</i>	miR-125a-3p	1.23 (0.69)	1.52 (0.24)	0.56
<i>ZMPSTE24</i>	miR-125a-3p	1.36 (0.69)	2.54 (1.38)	0.28
Skin fibroblasts				
<i>ADAMTS5</i>	miR-15b	0.99 (0.61)	1.47 (0.44)	0.33
<i>ADCY3</i>	miR-92a	1.07 (0.12)	1.42 (0.40)	0.26
<i>CCDC28A</i>	miR-15b	0.86 (0.24)	1.22 (0.14)	0.13
<i>FAM134C</i>	miR-15b	0.97 (0.10)	1.24 (0.26)	0.2
<i>FRY</i>	miR-92a	1.22 (0.46)	1.72 (0.25)	0.2
<i>INMT</i>	miR-125a-3p	0.86 (0.28)	3.65 (0.98)	0.03
<i>IVNS1ABP</i>	miR-125a-3p	0.9 (0.26)	0.96 (0.21)	0.77
<i>LHFPL2</i>	miR-92a	0.87 (0.33)	1.58 (0.30)	0.05
<i>LYST</i>	miR-92a	0.83 (0.24)	2.11 (0.27)	0.02
<i>SLC36A1</i>	miR-15b	1.2 (0.42)	1.81 (0.31)	0.12
<i>TCFL5</i>	miR-92a	1.31 (0.55)	1.23 (0.29)	0.84
<i>TEX261</i>	miR-125a-3p	0.98 (0.23)	1.3 (0.21)	0.16
<i>ZFP36L1</i>	miR-125a-3p	1.07 (0.22)	1.21 (0.32)	0.55
<i>ZMPSTE24</i>	miR-125a-3p	0.97 (0.15)	1.58 (0.18)	0.01

Mean expression values are given for each target gene in early passage (non-senescent) and late passage (senescent) lung and skin fibroblasts by qRT-PCR. RQ = Relative quantification. Figures in brackets are the standard deviation of measurement. Statistical significance assessed by *t* test are given for each target. Significant results are indicated in bold type. The miRNA predicted to target each transcript is given. The five genes not expressed in these cell types (*CDH10*, *MAL2*, *SMYD4*, *TACC2*, *TPPP*) have been omitted from the table

Table 4

Association between age and bioinformatically-predicted target gene expression in peripheral blood from a population study of aging

Gene	Targeted by	β coefficient	95 % CI	FDR adjusted q value
<i>ADAMTS5</i>	hsa-miR-15b	Not expressed		
<i>ADCY3</i>	hsa-miR-92a	Not expressed		
<i>CCDC28A</i>	hsa-miR-15b	-0.0012	-0.002 to -0.0004	0.02
<i>CDH10</i>	hsa-miR-92a	Not expressed		
<i>FAM134C</i>	hsa-miR-15b	Not expressed		
<i>FRY</i>	hsa-miR-92a	-0.0001	-0.0003 to 0.0001	0.4
<i>INMT</i>	hsa-miR-125a-3p	Not expressed		
<i>IVNS1ABP</i>	hsa-miR-125a-3p	-0.0005	-0.0009 to 2.8×10^{-6}	0.1
<i>LHFPL2</i>	hsa-miR-92a	0.0004	0.0002 to 0.0007	0.001
<i>LYST</i>	hsa-miR-92a	-0.0007	-0.001 to -0.0002	0.03
<i>MAL2</i>	hsa-miR-125a-3p	Not expressed		
<i>SLC36A1</i>	hsa-miR-15b	-0.0001	-0.0004 to 0.0002	0.4
<i>SMYD4</i>	hsa-miR-125a-3p	-0.0002	-0.0005 to 1.1×10^{-5}	0.1
<i>TACC2</i>	hsa-miR-92a	Not expressed		
<i>TCFL5</i>	hsa-miR-92a	-0.0001	-0.0004 to 0.0002	0.4
<i>TEX261</i>	hsa-miR-125a-3p	0.0001	-6.8×10^{-5} to 0.0003	0.2
<i>TPPP</i>	hsa-miR-92a	Not expressed		
<i>ZFP336L1</i>	hsa-miR-125a-3p	-0.0002	-0.0004 to -1.6×10^{-6}	0.09
<i>ZMPSTE24</i>	hsa-miR-125a-3p	-0.0012	-0.002 to -0.0004	0.02

We identified bioinformatically-predicted targets of MicroRNAs differentially expressed in both skin and lung fibroblasts and assessed potential associations with age in the InCHIANTI population study using fully adjusted multivariate linear regression. N = number of individuals in the analysis and FDR = False discovery rate. Results are given below. Results falling below an FDR adjusted q-value of 0.05 were considered significant. β = beta coefficient, SE = standard error. Significant results are indicated in bold type