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Pathway analysis of senescence-associated miRNA targets reveals common processes to different senescence induction mechanisms

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Centre for Oncology and Applied Pharmacology, University of Glasgow, Cancer Research UK Beatson Laboratories, Gartcube Estate, Switchback Road, Bearsden, Glasgow G61 1BD, UK.

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Requests for reprints: W. Nicol Keith, Centre for Oncology and Applied Pharmacology, University of Glasgow, Cancer Research UK Beatson Laboratories, Alexander Stone Building, Gartcube Estate, Switchback Road, Bearsden, Glasgow G61 1BD, United Kingdom. Phone: 44 141-330-4811; FAX: 44 141-330-4127; e-mail: n.keith@beatson.gla.ac.uk

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Summary

Multiple mechanisms of senescence induction exist including telomere attrition, oxidative stress, oncogene expression and DNA damage signalling. The regulation of the cellular changes required to respond to these stimuli and create the complex senescent cell phenotype has many different mechanisms. MiRNAs present one mechanism by which genes with diverse functions on multiple pathways can be simultaneously regulated. In this study we investigated 12 miRNAs previously identified as senescence regulators. Using pathway analysis of their target genes we tested the relevance of miRNA regulation in the induction of senescence. Our analysis highlighted the potential of these senescence-associated miRNAs (SA-miRNAs) to regulate the cell cycle, cytoskeletal remodelling and proliferation signalling logically required to create a senescent cell. The reanalysis of publicly available gene expression data from studies exploring different senescence stimuli also revealed their potential to regulate core senescence processes, regardless of stimuli. We also identified stimulus specific apoptosis survival pathways theoretically regulated by the SA-miRNAs. Furthermore the observation that miR-499 and miR-34c had the potential to regulate all 4 of the senescence induction types we studied highlights their future potential as novel drug targets for senescence induction.

1. Cellular senescence

The irreversible cell-cycle arrest known as senescence can be induced by a number of factors including: telomere attrition, known as replicative senescence [1]; oxidative stress [2]; oncogene expression [3] and DNA damage signalling [4]. Senescence is involved in the regulation of cellular aging and tissue maintenance and acts as a barrier to immortality and tumorigenesis. Individual regulatory mechanisms promoting the establishment of senescence have been characterised, including for example gene expression changes in p16, p53 and p21 [5]; chromatin silencing of E2F target genes [6] and protein phosphorylation by DNA damage checkpoint kinases [7]. All of these molecular changes occur in the larger cellular context and result in changes to pathways regulating different cellular processes, as summarised in Figure 1.

Complex cell phenotypes such as senescence are unlikely to result simply from a change in a single pathway but instead from cooperative changes in multiple systems. The complete network of underlying pathways resulting in cellular senescence is currently unclear. Investigations of mechanisms by which senescence is regulated continue to present novel pathways and regulatory molecules involved in the process. MicroRNAs (miRNAs) present a mechanism by which genes involved in a variety of different signalling pathways can be regulated simultaneously. They therefore present a further level of molecular interactions by which senescence may be induced.

1.1 MiRNAs as potential regulators of cellular senescence

Of the many ways to regulate gene product expression, miRNAs are relatively poorly understood. These short non-coding molecules ranging in size from 19-22 nucleotides are highly conserved and regulate protein expression through interactions with the 3' untranslated region (UTR) of mRNA [8, 9]. The binding of a miRNA to the 3'UTR causes inhibition of translation through steric hindrance or degradation of the mRNA, depending on the degree of complementarity between the two sequences [10]. The ability of miRNAs to regulate a variety of target genes allows them to induce changes in multiple pathways and processes such as development [11], apoptosis, proliferation and differentiation [12]. MiRNAs could therefore facilitate the complex cellular changes required to establish the senescent phenotype.

Identification of the mRNA sequences that miRNAs regulate is mainly derived using bioinformatics techniques. The ability of each miRNA to target genes is based on sequence complementarity to 3'UTRs of known mRNAs. The mirBase sequence database is the main repository for miRNA sequence and target information and (as of the 18 Nov 2008) contains 695 human miRNA sequences, each with the potential to regulate on average 1000 gene targets [13-15]. It is this large number of potential targets across different biological pathways that give miRNAs the power to potentially induce complex cell phenotypes, like senescence.

2. Twelve senescence associated miRNAs with experimental evidence for regulation of senescence

Although the gene targets of miRNAs can be identified theoretically by computational algorithms, it is unclear exactly which potential target genes they modulate and to what extent this affects the associated functions of these genes. The identification of miRNAs with the ability to regulate a complex phenotype, like senescence, is therefore not possible using purely bioinformatic techniques. For this reason we performed database searches of the terms miRNA and senescence, using pubmed and ISI web of knowledge (accessed 18 Nov 2008), to identify miRNAs with experimental evidence of involvement in senescence. The literature search highlighted 6 studies with direct evidence for regulation of senescence by miRNAs. Two main approaches were used in these studies: microarray based and comprehensive functional genomics; and the investigation of candidate senescence genes and the miRNAs regulating them. The 6 studies highlight a total of 12 senescence-associated miRNAs (SA-miRNAs). Each miRNA has the ability to bind between 953 and 2351 sites on mRNA 3' UTRs (see table 1) and in addition some 3'UTRs potentially have multiple complementary sites for a given miRNA. To add to the complexity, in reality this set of miRNAs may be a subset of the total number of miRNAs that play a role in senescence.

Looking at the individual SA-miRNAs we can see that some miRNAs have more support for their roles in senescence than others (Table 1). The evidence for a regulatory role of the miR-34 family of miRNAs in senescence is growing

and has stemmed from the investigation of p53 and its role in senescence. A number of studies have identified the miR-34 family, particularly miR-34a, as downstream effectors of p53 involved in the cell cycle [16]. Recent research has found that up-regulation of miR-34a in various cancer cell lines leads to cell cycle arrest, increased expression of β -galactosidase [17] and down-regulation of E2F family target genes [18]. Furthermore, drug treatment of various cancer cell lines with the MDM2 inhibiting drug Nutlin-3, leading to p53 activation, induced up-regulation of primarily miR-34a and later miR-34b and miR-34c [19]. Reports of a pro-apoptotic effect induced by miR-34a [20, 21] may demonstrate that its expression alone is not responsible for senescence.

The potential role of hsa-miR-20a in senescence regulation was discovered through investigation of the candidate senescence associated gene LRF [22]. LRF is a transcriptional repressor of the MDM2 inhibitor p19ARF [23, 24]. The overexpression of miR-20a in mouse embryonic fibroblasts induced senescence by lowering LRF protein levels and in turn increasing p19ARF levels. The fact that investigation of two different senescence-associated genes, p53 and LRF, yields different SA-miRNAs may be a reflection of the range of signalling pathway changes that these molecules contribute to the induction of the senescent phenotype.

Genome-wide analysis of changes in miRNA expression or investigations of those miRNAs that allow senescence bypass are alternative approaches that have been used in SA- miRNA identification. The use of microarrays identified

miRNAs hsa-miR-371, hsa-miR-369-5p, hsa-miR-29c, hsa-miR-499 and hsa-let-7f as significantly up-regulated in senescent human mesenchymal stem cells (hMSCs) when compared to early passage hMSCs [25]. MiRNA hsa-miR-271 was also seen to be significantly up-regulated by this study but overall had very low expression levels. These varying levels of miRNA expression may suggest different requirements for alterations in their target gene pathways.

MiR-372 and miR-373 were identified as miRNAs whose expression was able to bypass RAS-induced senescence in the presence of wild-type p53 [26]. The expression of these miRNAs would therefore not be conducive to senescence and we include them in our analysis as examples of miRNAs whose targets may increase in expression during senescence.

These reports provide experimental evidence for the molecular mechanisms by which miRNAs may act in the context of senescence. Recent reports of the regulatory effects of miRNAs miR-17-5P and miR-24 on senescence regulators p21 and p16, may offer further miRNAs with a role in senescence [27, 28].

However, lack of β -galactosidase and γ H2AX analysis or the expected alterations to senescence induction prevent us from being completely clear whether these miRNAs play a direct role in senescence. For this reason we did not include miR-17-5P or miR-24 in our SA-miRNA cohort. By examining the biological pathways that the 12 SA-miRNAs named in Table 1 can theoretically regulate, we aim to gain a greater understanding of the biological changes that these miRNAs could create that potentially lead a cell into senescence.

2.1 Pathway mapping of senescence associated miRNA targets shows cell cytoskeletal, cell cycle and proliferation regulation potential

By mapping of the signalling pathways regulated by the SA-miRNAs we aimed to better understand the biological mechanisms by which they can cooperate to create the complex senescence phenotype. The free, publicly available Sanger miRNA database contains a registry of computationally calculated gene targets for each known miRNA. We downloaded (25 Aug 2008) and queried the current version of the database (mirBase targets v5) using Microsoft Access, in order to generate lists of theoretical gene targets for the 12 SA-miRNAs. As the computational method of assigning gene targets to miRNAs cannot assess the level of complementarity required for a biological effect, we decided to include all theoretical targets in our analysis.

The signalling pathways and processes that these gene targets are involved in were explored using the systems biology tool Metacore from Genego (Genego Inc, St Joseph, MI). This database of known molecular interactions, pathways and processes manually curated from published data allows the user to visualise known biological systems within their data. Input of the SA-miRNA target gene lists into the Metacore database allowed us to explore the potential biological implications of their regulation during the induction of senescence.

The top 5 pathways regulated by the SA-miRNAs are shown in Table 2. Two of these maps are involved in cytoskeletal remodelling and are highly saturated with gene targets of the SA-miRNAs (Figure 2). Senescent cells are seen to undergo changes in morphology, becoming large and flattened [29], which would require significant cytoskeletal remodelling. Such widespread cellular modifications would be associated with the alteration of many different pathways and processes that all contribute to the maintenance of the actin cytoskeleton. Due to their ability to bind a number of different gene targets miRNAs provide a mechanism by which such widespread changes could be induced.

Changes in the processes involved in DNA synthesis in early S-phase are also potentially regulated by the SA-miRNAs (Figure 3). Of the objects on this map 59% are potentially regulated by the SA-miRNAs and in particular members of the MCM and ORC complexes. The ORC and MCM complexes are responsible for the initiation of DNA replication by assembly of the pre-replication complexes and ensure that the process only occurs once during the cell cycle [30]. Recently the ORC complex was reported to be able to interact with the telomere binding protein TRF2 and affect telomere homeostasis [31].

Replicative senescence relies on the shortening of telomeres to induce a DNA damage checkpoint response that blocks their progression into S-phase [7]. Since senescence involves irreversible cell cycle arrest, SA-miRNA regulation of cell cycle mechanisms strengthens their possible role in senescence regulation.

The transcription factor CREB is also potentially regulated by SA-miRNAs (Figure 4). CREB is a known regulator of cellular growth and proliferation through its transcriptional regulation of genes such as the oncogene *c-fos* [32] and regulators of the cell cycle such as cyclin D1 [33] and cyclin A [34]. Alteration of molecules regulating CREB by SA-miRNAs would therefore confer another mechanism to alter the cell growth and proliferation pathways towards a senescent state.

The regulation of EGFR and its proliferative signalling is not conducive to a senescent cellular environment. Consistent with this, EGFR is repressed at the chromatin level during senescence [6] and is shown to be potentially regulated by the 12 SA-miRNAs (Table 2). The ability of SA-miRNAs to further repress these signals provides a cell entering senescence another regulatory mechanism by which to ensure their silencing.

The multiple pathway alterations discussed suggest that these SA-miRNAs are potentially regulating many of the necessary steps required to create a fully senescent cell, from changes in the cellular cytoskeleton to alteration of multiple pathways regulating cell cycle and proliferation signals. Their potential to act as master regulators of senescence could be a prospective source of novel drug targets and biomarkers. Senescence can be stimulated by more than one mechanism however, including induction by telomere attrition (replicative senescence), oncogenes, drugs and cellular stress. It remains unclear whether the same pathway alterations and miRNAs are involved in each of the different stimuli or whether each stimulus takes a

different route to the same endpoint of a senescent cell, as depicted in Figure 5.

3. Investigation of SA-miRNA regulation of different senescence stimuli

In order to investigate the potential of SA-miRNAs to regulate the different senescence stimuli we returned to the literature. Using previously published data we queried differentially expressed gene lists from replicative senescence in hMSCs [25], drug induced replicative senescence by a telomerase inhibitor [35], cellular stress by Ethanol or *tert*-butylhydroperoxide exposure [2] and oncogene induced senescence by BRAF^{E600} [3].

Each of the datasets queried showed some potential to be regulated by the SA-miRNAs as shown in Table 3. Using the results of these 4 analyses, we explored the potential of SA-miRNAs to regulate pathways with gene expression changes for each stimulus. The differences and similarities in SA-miRNAs and their targeted pathways between stimuli were also investigated.

3.1 Pathways regulated by SA-miRNAs during replicative senescence show increased cell survival and decreased cell cycle signalling

By reanalysing the publicly available gene expression data from the study by Wagner and colleagues, we examined the pathways during replicative senescence that could be regulated by SA-miRNAs (Table 4a) [25]. Gene lists

of 2 fold or more changes in expression between early and late passage hMSCs were constructed. Filtering of these lists to only those genes theoretically regulated by the SA-miRNAs led to the construction of 2 lists: 85 gene targets with increased expression and 49 gene targets with expression in late passage compared to early passage hMSCs. The pathways associated with each gene list were then individually explored using Metacore.

Looking initially at the pathways that have gene targets with increased expression in Table 4a, we see that 3 of the 5 maps on the list are involved with apoptosis and survival through the FAS and TNFR1 signalling pathways or in a p53 dependent manner. In all 3 maps the only molecules that the SA-miRNAs are regulating are BRE and c-FLIP. BRE is known to suppress the apoptotic pathways through binding and inhibition of the FAS receptor [36] and the TNF-alpha receptor [37]. C-FLIP is a potent inhibitor of apoptosis through its binding and resulting inhibition of the adaptor protein FADD [38]. Although the SA-miRNAs are only regulating 2 molecules involved in the survival and apoptosis signalling pathways, regulation of the absolute levels and timing of such signals may be one of the mechanisms by which the SA-miRNAs ensure the cells enter senescence rather than apoptosis.

The second most significant pathway with increases in gene expression during replicative senescence is cytoskeletal remodeling centering on the role of intermediary filaments. As discussed previously, changes in the cellular morphology of senescent cells would require significant changes to the cytoskeleton of the cell. Increases in expression of key genes regulating many

of these processes would therefore be required. The SA-miRNAs provide one potential mechanism by which these changes could be regulated.

Changes to the immune response and the IL1 signaling pathway during replicative senescence are also potentially regulated by the SA-miRNAs. The concept of the involvement of IL1 and other pro-inflammatory networks in the induction of senescence is concurrent with current findings [39-41]. Therefore the ability of SA-miRNAs to regulate this pathway demonstrates a further mechanism by which they could induce senescence.

The regulation of cellular survival, pro-inflammatory and cytoskeletal remodeling cellular processes would all logically be required to create the senescent cell phenotype. These processes can theoretically be regulated by the SA-miRNAs, during replicative senescence, showing that they have the potential to regulate the absolute levels and timings of these complex signaling cascades to create a senescent cell.

However, these are not the only pathways that the SA-miRNAs can regulate during this process. Four out of the 5 pathways that contain SA-miRNA gene targets with decreases in expression are involved in various aspects of cell cycle regulation (Table 4a). These include DNA replication in early S-phase and the roles of APC, the SCF complex and ESR1. Regulation of key molecules in the multiple processes governing the cell cycle during senescence induction is critical. The SA-miRNAs regulate a subset of molecules (CDK2, EMI1, Cyclin B, SKP2, and CDC18L) that are involved in regulation of these

multiple cell cycle pathways. This allows the SA-miRNAs to affect the cell cycle regulatory process as whole rather than at just one checkpoint or stage. The 12 SA-miRNAs that we have highlighted therefore exemplify the potential of miRNAs to play a regulatory role in this aspect of replicative senescence.

The remaining pathway containing gene targets down-regulated during replicative senescence is involved in dCTP/dUTP metabolism. Since senescent cells are no longer cycling, changes in metabolism will follow. SA-miRNAs may only be regulating a small part of cellular metabolism, the metabolism of 2 nucleotide units, however these metabolic changes may have far reaching effects. Furthermore, changes in dATP, ATP and TTP metabolism are seen further down in the list of pathways, 6, 7 and 9 respectively, which are not discussed here. It may therefore be the case that the SA-miRNAs are able to regulate the changes in metabolism required to establish senescence or that only changes to a few molecules or processes are required.

Changes in gene expression during the establishment of replicative senescence in hMSCs are potentially being regulated by the SA-miRNAs. We have observed that the SA-miRNAs targeted down-regulation of cell cycle pathways and increased signaling by pathways involved in cytoskeletal remodeling and apoptosis survival. The ability of a relatively small number of SA-miRNAs to differentially regulate such diverse changes demonstrates their potential in the induction of complex cellular phenotypes.

3.2 Regulation of drug induced senescence by SA-miRNAs also highlights increased cell survival and decreased cell cycle signalling

Replicative senescence results from the shortening of telomeres during each round of the cell cycle [1]. Many cancer cells reactivate the enzyme telomerase, which has the ability to maintain telomeres and therefore overcome this barrier to tumorigenesis [42]. Application of a drug that inhibits telomerase would, in theory, activate the cells' natural senescence pathways, potentially including the actions of the SA-miRNAs. To explore this hypothesis we interrogated the differentially expressed genes published by Damm and colleagues for potential SA-miRNA regulation [35].

Damm and coworkers investigated senescence resulting from treatment of lung carcinoma cells with the telomerase inhibitor BIBR1532. We filtered the published genes lists to include only those genes regulated by the 12 SA-miRNAs. This resulted in a list of 76 down-regulated SA-miRNA gene targets and 49 up-regulated gene targets (Table 4b). Pathway analysis was then performed to investigate the potential effects of SA-miRNA regulation in drug induced replicative senescence.

As seen previously during the analysis of replicative senescence, the pathways associated with gene targets showing increased expression involve a variety of different cellular mechanisms. Two of these pathways are involved in the regulation of cellular signalling by the molecules PTEN and

Activin A. The dual specificity phosphatase PTEN is a tumour suppressor gene and regulates cellular proliferation and survival through its ability to bind p53 in the nucleus [43]. Activin A is a member of the TGF β superfamily and is involved in the regulation of cell growth and apoptosis [44]. Both molecules are commonly deregulated in cancers [45, 46] reflecting their importance in regulation of cell proliferation processes. Alterations in these signalling pathways could therefore be beneficial to the establishment of a senescent cell phenotype. The theoretical regulation of these processes by the SA-miRNAs demonstrates further mechanisms by which SA-miRNAs can cause the complex signalling pathway changes required to induce senescence.

Two further pathways associated with gene expression increases that could be regulated by the SA-miRNAs involve EGFR and the ERBB family. As previously discussed, regulating signalling from the ERBB family of tyrosine kinases is an important step in senescence induction due to their role in the regulation of cellular proliferation. The presence of this map demonstrates that SA-miRNAs are a potential regulatory mechanism of these pathways during drug induced replicative senescence as well as natural replicative senescence.

The remaining pathway associated with increased gene target expression is involved with changes in metabolism regulated by the transcription factor AP-1. Closer inspection of the SA-miRNA targets on this map (Figure 6) highlights the role of AP-1 in regulation of p21 and the apoptosis molecules *c-fos* and FASR. The involvement of *c-fos* and FASR in pathways regulated by

the SA-miRNAs has been highlighted earlier in this study. This demonstrates that regulation of the diverse cellular proliferation and apoptosis signalling pathways affected by these molecules is an important step in senescence establishment, under both natural replicative senescence and when artificially drug induced. SA-miRNA regulation could therefore provide a mechanism by which the absolute levels and timings of these signals can be controlled.

The biological pathway map analysis of the decreases in gene expression for telomerase inhibited cells returned results very similar to that of the down-regulated genes in natural replicative senescence. Four of the 5 maps are involved with the regulation of various aspects of the cell cycle including the role of APC, the DNA damage response checkpoint, regulation of ESR1 and chromosome condensation in prometaphase (Table 4b). As with the cell cycle pathways seen in replicative senescence, the regulation of cell cycle following telomerase inhibition would be a critical step in senescence induction. Targeting these multiple pathways to cell cycle control give SA-miRNAs comprehensive regulation of the process.

The remaining map is involved with metabolism of folic acid. As previously discussed, changes in metabolism will be required in cells undergoing senescence. However, this may also reflect metabolic changes involved in the metabolism of BIBR1532, independent of senescence, that the SA-miRNAs are also able to regulate. The ability of SA-miRNAs to target not just the

down-regulation cell cycle aspects of senescence but also the metabolic changes demonstrates the flexibility and complexity of their action.

Looking at the pathway analysis of both replicative and drug induced senescence shown in Table 4, highlights the fact that the down-regulated pathways in both cases are cell cycle and metabolism related whilst the up-regulated pathways differ between the two stimuli. However, looking at the functions of the up-regulated pathways in both cases reveals a common apoptosis regulatory signal. Cells in replicative senescence appear to be signalling apoptosis survival through FAS and TNFR1 pathways or in a p53 dependant manner whereas the telomerase inhibited cells are using the actions of Activin A and PTEN for this purpose. Thus the SA-miRNAs are potentially using different cellular mechanisms by which to achieve the same goal. The SA-miRNA regulation of the cellular signals behind drug induced and natural replicative senescence are therefore stimuli specific whilst remaining focused towards the common outcome of creating the complex senescent cell phenotype.

3.3 SA-miRNA regulation of oncogene induced senescence emphasises the importance of inflammatory signals in senescence

Telomere erosion leading to the activation of DNA damage signalling cascades is not the only method of senescence induction. Oncogene induced senescence (OIS) can be caused upon either the loss of tumour suppressor genes like PTEN [47] or activation of oncogenes such as MEK, RAS and BRAF^{E600} [48-50]. Recent evidence suggests that senescence induction by

these stimuli is dependent upon IL-6 mediated inflammatory responses without which the cells bypass senescence and continue proliferating [3]. The same study also used gene expression microarrays to examine differentially expressed genes between cells that bypass and cells that undergo OIS, revealing a signature of 20 genes shown in Table 5. Seven of these genes, including IL-6, can theoretically be regulated by SA-miRNAs.

Due to the small size of the initial signature, and hence the small number of target genes, analysis of the biological pathways underlying the SA-miRNA regulation was not statistically feasible. However, the SA-miRNAs regulate nearly 30% of the OIS signature revealed by Kuilman *et al*, including the key molecule IL-6 (Table 5). This may demonstrate the principle that large numbers of gene alterations are not always required for senescence induction, just well targeted ones. The multiple regulation of 3 of the genes in particular (C20orf26, CD55 and IL6) by the SA-miRNAs may show that within this signature these genes require the strictest regulation to ensure the successful induction of senescence, although further validation is required to test this theory.

Our analysis of differentially expressed genes during oncogene induced senescence, although limited by the size of gene list, still highlights the potential of SA-miRNAs to regulate key molecules required for this form of senescence induction. Furthermore the regulation of inflammatory networks shown in this study to be key to senescence induction was also highlighted during our analysis of replicative senescence. This common regulation of pro-

inflammatory genes in response to 2 different senescence stimuli by SA-miRNAs is suggestive of its importance in senescence as a whole.

3.4 Regulation of stress induced senescence by SA-miRNAs transcends treatment type to control core senescence processes

Senescence can also be induced by many different types of cellular stress such as UV-B [51], hydrogen peroxide [52], *tert*-butylhydroperoxide (*t*-BHP) or ethanol exposure [53]. Pascal and colleagues used a combination of differential display RT-PCR and low density DNA arrays to investigate genes involved in senescence induction by ethanol or *t*-BHP [2]. They revealed signatures of genes increasing and decreasing in expression for both ethanol and *t*-BHP, many of which overlapped. Table 6 highlights the potential of the SA-miRNAs as regulators of stress induced senescence by both treatments. 75% of the genes theoretically regulated by SA-miRNAs overlap between the two treatment types. This may suggest that these target genes are those that are core to senescence induction by SA-miRNA regulation.

Of all the overlapping genes shown in Table 6 four have roles in regulation of the actin cytoskeleton, namely ARHGAP24, ARPC2, MACF1 and S100A4 [54-56]. In our previous analysis cytoskeletal remodelling has always been associated with genes showing increased expression levels. Here however two of the molecules, ARHGAP24 and ARPC2 show decreased expression. This may therefore represent specific molecules that require negative regulation in the context of stress induced senescence. Alternatively this may represent down-regulation of genes required to create the necessary senescence-

associated cytoskeletal alterations. This demonstrates the potential of SA-miRNAs to regulate similar processes through different mechanisms dependant on stimuli.

Cell cycle regulation has in both replicative and drug induced senescence been strongly negatively regulated. Two of the overlapping down-regulated genes, RAD17 and CDC37L1 (Table 6), common to both ethanol and *t*-BHP treatments have roles in the cell cycle. RAD17 specifically is involved in cell cycle arrest caused by DNA damage signalling [57]. The DNA damage caused by reactive oxygen species in cellular senescence may therefore be activating similar mechanisms of senescence induction as that seen in replicative senescence. These gene targets highlight the fact that the SA-miRNAs are able to induce similar mechanisms through the use of stimuli specific target genes. The flexibility of the SA-miRNA mechanism of action allows them to remain specific enough for a cell to respond to a particular stimuli whilst general enough to focus pathway changes in those cells to create the senescent cell phenotype.

Two of the overlapping genes, EWSR1 and LOXL2, have previous evidence of senescence association. The EWS fusion protein has recently been reported as a repressor of senescence [58] and therefore its down-regulation by SA-miRNAs would provide a mechanism by which to prevent it inhibiting senescence induction. LOXL2 however has been used as a marker of senescent cells due to its increased expression during senescence [59]. These senescent associated genes can theoretically be regulated by SA-miRNAs and

this may therefore present a mechanism by which their roles in senescence induction are controlled.

The remaining overlapping genes may still have roles in senescence. These genes may be involved in senescence induction through previously identified or potentially novel unidentified pathways, which analysis of the individual gene function does not elucidate. Their inclusion into larger data sets of potential senescence associated genes for systems biology analysis may help to elucidate their role.

MiRNA regulation of stress induced senescence can therefore be seen to potentially transcend treatment type and incur changes in core senescence genes. Furthermore regulation of additional treatment specific genes gives SA-miRNAs the potential to alter diverse cellular pathways required to respond to different stimuli.

4. SA-miRNAs regulate common cellular mechanisms regardless of stimuli

The regulation of different senescence induction stimuli by miRNAs varies as shown in Table 3. In this study we have seen evidence of the potential SA-miRNAs regulation of core sets of senescence genes regardless of induction mechanism. Our analysis has highlighted common up-regulation of apoptosis signalling and pro-inflammatory gene regulation targeted by SA-miRNAs between different senescence stimuli. We have also seen SA-miRNA targeted

down-regulation of cell cycle processes across the induction mechanisms. This evidence taken in combination with the large degree of overlap between treatment types in stress induced senescence is suggestive of key cellular changes that the SA-miRNAs can facilitate during senescence induction. To our knowledge, this is the first report of predicted miRNA regulation of common pathways regardless of senescence stimulus.

The fact that 2 SA-miRNAs, hsa-miR-499 and hsa-miR-34c, can regulate all of the senescence types we have examined takes this concept one step further and may suggest that these particular SA-miRNAs act as master regulators for senescence induction. Alterations in different biological signaling pathways by the remaining 10 SA-miRNAs could then tailor the cellular response to suit the alterations required by specific stimuli. This hypothesis requires further laboratory validation, however the fact that both miRNAs already have experimental evidence to show they are senescence regulators adds some weight to the theory.

5. Conclusions

In this study we have used pathway mapping and theoretical gene target identification to create a biological framework by which to test the relevance of miRNAs in senescence induction. We have shown not only that miRNAs can potentially regulate genes previously seen to be involved in senescence but also regulate larger pathway alterations, such as cell cycle and cytoskeletal remodeling, that would logically be required to create the

complex phenotype of the senescent cell. Commonly regulated pathways and cellular mechanisms between the senescence stimuli also demonstrate the potential of SA-miRNAs to regulate a core set of pathway modifications regardless of senescence induction mechanism. Furthermore, we have identified stimulus specific pathways by which SA-miRNAs can regulate apoptosis survival signals in cells undergoing drug induced or natural replicative senescence. The identification of miR-499 and miR-34c as common regulators of all senescence stimuli we have studied may highlight their potential as future drug targets for senescence induction and further investigation of their role in regulation of the senescence phenotype certainly warrants further investigation.

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Figure Legends

Figure 1 - The establishment of a complex cell phenotype, such as senescence, requires the interaction of many regulatory mechanisms leading to changes in individual pathways and processes.

Figure 2 - SA-miRNA regulation of the Cytoskeletal remodelling and the role of TGF and WNT biological pathway map. Red thermometers show an object that can be regulated by a SA-miRNA with thermometer numbering corresponding to that seen in Table 1. Multiple thermometers denote that the object is a target of multiple SA-miRNAs. Green arrows represent positive, red negative and grey unspecified interactions. Boxes on lines denote the type of regulation where P = phosphorylation; B = binding; Tr = transcriptional regulation, Cm = covalent modification, T = transformation and Z = catalysis.

Figure 3 - SA-miRNA regulation of Cell cycle and DNA replication in early S phase biological map. Red thermometers show an object that can be regulated by a SA-miRNA with thermometer numbering corresponding to that seen in Table 1. Multiple thermometers denote that the object is a target of multiple SA-miRNAs. Green arrows represent positive, red negative and grey unspecified interactions. Boxes on lines denote the type of regulation where P = phosphorylation; B = binding; Tr = transcriptional regulation, Cm = covalent modification, T = transformation and Z = catalysis.

Figure 4 - SA-miRNA regulation of Transcription and the CREB pathway biological map. Red thermometers show an object that can be regulated by a SA-miRNA with thermometer numbering corresponding to that seen in Table 1. Multiple thermometers denote that the object is a target of multiple SA-miRNAs. Green arrows represent positive, red negative and grey unspecified interactions. Boxes on lines denote the type of regulation where P = phosphorylation; B = binding; Tr = transcriptional regulation, Cm = covalent modification, T = transformation and Z = catalysis.

Figure 5 - Potential mechanisms of miRNA regulation in Senescence and their potential. A small subset of miRNAs, such as the 12 SA-miRNAs that we have identified, may be acting as master regulators of the senescent phenotype. However it is also possible that individual types of senescence induction may require different miRNAs to create the same cellular changes due to the different stimuli and therefore signalling pathways involved. Overlaps in the biological pathways or miRNAs could therefore be utilised to discover novel drug targets for senescence induction.

Figure 6 - SA-miRNA regulation of transcription and the role of AP1 in regulation of cellular metabolism pathway biological map during drug induced senescence. Red thermometers show an object that can be regulated by a SA-miRNA with thermometer numbering corresponding to that seen in Table 1. Multiple thermometers denote that the object is a target of multiple SA-miRNAs. Green arrows represent positive, red negative and grey

unspecified interactions. Boxes on lines denote the type of regulation where

P = phosphorylation; B = binding; Tr = transcriptional regulation, Cm =

covalent modification, T = transformation and Z = catalysis.

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Tables

Number	Senescence Associated miRNA	Number of predicted complementary sites in the transcriptome	References
1	hsa-let-7f	1018	Wagner <i>et al.</i> , 2008
2	hsa-miR-499	2157	Wagner <i>et al.</i> , 2008
3	hsa-miR-373	1057	Voorhoeve <i>et al.</i> , 2006
4	hsa-miR-372	953	Voorhoeve <i>et al.</i> , 2006
5	hsa-miR-371	2205	Wagner <i>et al.</i> , 2008
6	hsa-miR-369-5P	1107	Wagner <i>et al.</i> , 2008
7	hsa-miR-34c	2351	Kumamoto <i>et al.</i> , 2008
8	hsa-miR-34b	1086	Kumamoto <i>et al.</i> , 2008
9	hsa-miR-34a	1296	Tazawa <i>et al.</i> , 2007, He <i>et al.</i> , 2007, Kumamoto <i>et al.</i> , 2008
A	hsa-miR-29c	1199	Wagner <i>et al.</i> , 2008
B	hsa-miR-217	993	Wagner <i>et al.</i> , 2008
C	hsa-miR-20a	1275	Poliseno <i>et al.</i> , 2008

Table 1 - Senescence associated miRNAs from the literature and the number of computationally derived mRNA 3' untranslated regions each can target. (Note some mRNAs may contain multiple binding sites) Numbering denotes the thermometer number representing particular miRNA's targets on Figures 2, 3 and 4.

	Pathway Map	% of objects on the map regulated by SA-miRNAs
1	The role of TGF and WNT in Cytoskeletal remodelling	44% (49/111)
2	Cytoskeletal remodelling general process	42% (43/102)
3	Cell Cycle: The start of DNA replication in early S-phase	59%(19/32)
4	Regulation of transcription in the CREB pathway	50% (21/42)
5	Development EGFR signalling via small GTPases	41% (13/32)

Table 2 - The top 5 pathways regulated by all 12 SA-miRNAs with the percentage of the map that consists of SA-miRNA gene targets . Gene target lists for each SA-miRNA were input to Metacore and the pathway maps which were commonly regulated were examined. Pathways are ordered by the number of SA-miRNA targets on each map. The presence of 4 regulatory pathways involving different aspects of cellular signalling and only one cell cycle pathway shows that there is more to senescence than just cell cycle regulation. SA-miRNAs therefore present a mechanism by which a variety of the required cellular pathway changes could be regulated.

	Replicative senescence		Drug induced Replicative Senescence		Oncogene Induced Senescence	Stress induced senescence			
	UP 85 target genes	DOWN 49 target genes	UP 76 target genes	DOWN 49 target genes	7 target genes	ETOH UP 10 target genes	EtOH Down 6 target genes	tBHP Up 6 target genes	tBHP Down 6 target genes
SA-miRNA	miRNA	miRNA	miRNA	miRNA	miRNA	miRNA	miRNA	miRNA	miRNA
hsa-let-7f	✓	✓	✓	✓			✓		✓
hsa-miR-20a	✓	✓	✓	✓	✓		✓		✓
hsa-miR-217	✓	✓	✓	✓	✓		✓		✓
hsa-miR-29c	✓	✓	✓	✓	✓	✓		✓	
hsa-miR-34a	✓	✓	✓	✓			✓		✓
hsa-miR-34b	✓	✓	✓	✓	✓	✓	✓		✓
hsa-miR-34c	✓	✓	✓	✓	✓	✓	✓	✓	✓
hsa-miR-369-5P	✓	✓	✓	✓	✓	✓		✓	
hsa-miR-371	✓	✓	✓	✓	✓	✓		✓	
hsa-miR-372	✓	✓	✓	✓					
hsa-miR-373	✓	✓	✓	✓					
hsa-miR-499	✓	✓	✓	✓	✓	✓	✓	✓	✓

Table 3 - Regulation of gene expression signatures of different types of senescence induction. Ticks represent the ability of that SA-miRNA to theoretically regulate one or more genes within the signature. All four senescence induction signatures are regulated by at least 5 SA-miRNAs. These differences in SA-miRNA regulation may represent the differing need for regulation of SA-miRNA target genes by different stimuli. The regulation of all of the senescent signatures studied by microRNAs hsa-miR-499 and hsa-miR-34c may demonstrate their potential as regulators of core senescence genes required by all senescence stimuli.

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SA-miRNA	Replicative senescence					
	85 Upregulated SA-miRNA gene targets			49 Downregulated SA-miRNA gene targets		
SA-miRNA	Pathway Maps	% Regulated by SA-miRNAs	p-value	Pathway Maps	% Regulated by SA-miRNAs	p-value
1	Apoptosis and survival p53-dependent	9% (2/29)	9.58E-03	Cell Cycle Role of APC in cell cycle regulation	22% (7/32)	5.15E-11
2	Cytoskeletal remodeling Keratin Filaments	6% (2/36)	1.45E-02	Cell Cycle Start of DNA replication in early S-Phase	19% (6/32)	3.79E-09
3	Immune Response IL1 signaling pathway	5% (2/37)	1.53E-02	dCTP/dUTP metabolism	8% (6/75)	7.37E-07
4	Apoptosis and survival FAS signaling cascades	5% (2/43)	2.04E-02	Cell Cycle Role of SCF complex in cell cycle regulation	14% (4/29)	6.82E-06
5	Apoptosis and survival TNFR1 signaling pathway	5% (2/43)	2.04E-02	Cell Cycle Sister chromatid cohesion	14% (3/22)	1.13E-04

SA-miRNA	Drug induced Replicative Senescence					
	49 Upregulated SA-miRNA gene targets			76 Downregulated SA-miRNA gene targets		
SA-miRNA	Pathway Maps	% Regulated by SA-miRNAs	p-value	Pathway Maps	% Regulated by SA-miRNAs	p-value
1	Signal Transduction PTEN pathway	11% (5/46)	6.52E-07	Cell Cycle Role of APC in cell cycle regulation	16% (5/32)	1.26E-06
2	Development EGFR signaling via small GTPases	14% (4/28)	3.13E-06	Folic acid metabolism	9% (5/53)	1.63E-05
3	Signal Transduction Activin A signaling regulation	14% (4/28)	3.13E-06	DNA damage ATM/ATR regulation of G1/S checkpoint	13% (4/32)	3.98E-05
4	Transcription role of AP1 in regulation of cellular metabolism	10% (4/42)	1.65E-05	Cell Cycle ESR1 regulation of G1/S transition	12% (4/33)	4.51E-05
5	Development ERBB-family signaling	9% (4/43)	1.81E-05	Cell cycle chromosome condensation in prometaphase	14% (3/21)	2.68E-04

Table 4 - Top 5 biological pathway maps that contain SA-miRNA gene targets shown in order of p-value.

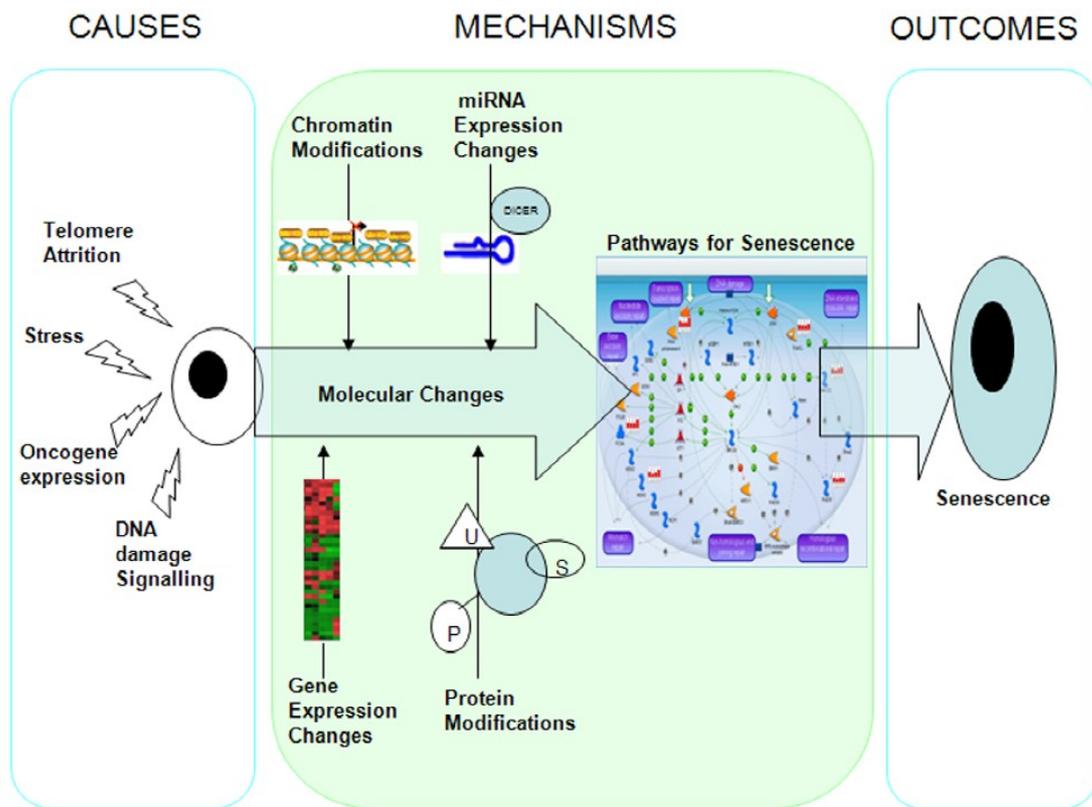
- (a) Wagner et al microarray data was reanalysed and all genes with a 2 fold or more difference between early passage and late passage hMSCs were selected. These lists were then filtered selecting only gene targets of the 12 SA-miRNAs and biological pathways maps analysed using Metacore from Genego.
- (b) Damm et al published differentially expressed gene signatures which were filtered selecting only gene targets of the 12 SA-miRNAs and biological pathways maps analysed using Metacore from Genego.

Gene name	Regulating SA-miRNAs
C20ORF26	hsa-miR-369-5P (2), hsa-miR-29c (1), hsa-miR-499 (3), hsa-miR-34b (1), hsa-miR-34c (2)
CD55	hsa-miR-29c (1), hsa-miR-34b (2), hsa-miR-34c (3)
CD9	
CPE	
FAM131A	
FAM43A	hsa-miR-371 (1)
GABRA2	
GEM	
GMFG	hsa-miR-369-5P (1)
IL6	hsa-miR-499 (1), hsa-miR-216 (1), hsa-miR-371 (1)
IQGAP2	
ITGA2	
ITPKA	
NR4A2	
PCNX	
PECAM1	
PTGES	hsa-miR-371 (1)
RPS6KA5	
TESC	hsa-miR-20a (1)
VGF	

Table 5 - 20 Differentially expressed genes upon induction of oncogene induced senescence highlighted by Kuilman *et al.* Also shown are the SA-miRNAs that can theoretically regulate 7 of these genes, brackets show the number of theoretical 3'UTR binding sites that each SA-miRNA has for that gene.

Gene	Effect of t-BHP treatment	Effect of ETOH treatment	Regulating SA-miRNAs
ARHGAP24	DOWN	DOWN	hsa-miR-499 (2)
ARPC2	DOWN	DOWN	hsa-miR-217 (2)
RAD17	DOWN	DOWN	hsa-let-7f (3)
			hsa-miR-20a (2), hsa-miR-34b (1), hsa-miR-499 (2), hsa-miR-34c (1)
CDC37L1	DOWN	DOWN	hsa-miR-20a (1)
EWSR1	DOWN	DOWN	hsa-miR-34a (2), hsa-miR-34c (2)
PHGDH	DOWN	DOWN	hsa-miR-369-5p (2)
MACF1	UP	UP	hsa-miR-369-5p (2)
RPS12	UP	UP	hsa-miR-29c (1)
LOXL2	UP	UP	hsa-miR-499 (2)
S100A4	UP	UP	hsa-miR-371 (1)
SLC35A5	UP	UP	hsa-miR-34c (1)
EEF1A1	UP	UP	hsa-miR-34c (1)
TBRG4	UP		hsa-miR-29c (1)
KPNB1	UP		hsa-miR-499 (1)
TMSB10	UP		hsa-miR-34b (2), hsa-miR-34c (1)
C21orf34	UP		

Table 6 - SA-miRNA targets showing alterations in gene expression after stress induced senescence by either *tert*-butylhydroperoxide (t-BHP) or Ethanol (EtOH) and the direction of their alteration, blank cells infer no effect. Also shown is the SA-miRNAs that can theoretically regulate these genes, brackets show the number of theoretical 3'UTR binding sites that each SA-miRNA has for that gene.



ACCEPTED

