miR-20a and miR-290, multi-faceted players with a role in tumourigenesis and senescence

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Abstract

Expression of microRNAs changes markedly in tumours and evidence indicates that they are causatively related to tumourigenesis, behaving as tumour suppressor microRNAs or onco microRNAs; in some cases they can behave as both depending on the type of cancer. Some tumour suppressor microRNAs appear to be an integral part of the p53 and Retinoblastoma (RB) network, the main regulatory pathways controlling senescence, a major tumour suppressor mechanism. The *INK4a/ARF* locus which codifies for two proteins, p19ARF and p16INK4a, plays a central role in senescence by controlling both p53 and RB. Recent evidence shows that the proto-oncogene leukaemia/lymphoma related factor, a p19ARF specific repressor, is controlled by miRNAs and that miRNAs, in particular miR-20a and miR-290, are causatively involved in mouse embryo fibroblasts (MEF) senescence in culture. Intriguingly, both miR-20a, member of the oncogenic miR-17–92 cluster, and miR-290, belonging to the miR-290–295 cluster, are highly expressed in embryonic stem (ES) cells. The pro-senescence role of miR-20a and miR-290 in MEF is apparently in contrast with their proliferative role in tumour and ES cells. We propose that miRNAs may exert opposing functions depending on the miRNAs repertoire as well as target/s level/s present in different cellular contexts, suggesting the importance of evaluating miRNAs activity in diverse genetic settings before their therapeutic use as tumour suppressors.

Keywords: miR-20a and miR-290 • senescence • tumour suppressors • mouse embryo fibroblasts • leukaemia/lymphoma related factor

Cellular senescence is a powerful tumour suppressor mechanism

A cell is defined as senescent when it fails to proliferate in response to growth factors while remaining alive for a long time. Senescent cells are phenotypically distinguishable from young cells in that they are characterized by a typical flat morphology and express specific biochemical markers such as pH-6 β-galactosidase, p16INK4a, p21CIP1, plasminogen activator inhibitor-1 (PAI-1) and phosphorylated H2AX [1–3].

The most studied *in vitro* models of senescent cells are human primary fibroblasts (HF) and mouse embryo fibroblasts (MEF). In the case of HF it is believed that the main stimulus is telomere shortening [3]. After 50–80 population doublings telomere shortening leads to uncapping of the chromosomal ends, which is recognized as double strand breaks leading to p53 activation with consequent cell cycle block and senescence [3]. This type of senescence is called intrinsic senescence to distinguish from telomere independent extrinsic senescence. HF can undergo extrinsic senescence when exposed to a variety of stress factors such as ionizing radiation, UV, DNA damaging drugs or reactive oxygen species [4]. MEF in culture mainly undergo extrinsic senescence, which takes place after about 10 population doublings, despite the presence of normal length telomeres. In this case the accumulation of reactive oxygen species during cell replication, with the consequent induction of cellular (DNA, protein) damage, appears to be responsible for senescence [5]. Oncogene activation, which induces DNA damage due to DNA hyper-replication [6], is a strong inducer of extrinsic senescence in both MEF and HF: in this case senescence represents a potent anti-tumourigenic mechanism protecting the organism from unwanted cell proliferation. Tumour suppressor mechanisms have evolved in organisms with renewable tissues in order to lower the probability of tumourigenesis but the same mechanisms might be responsible

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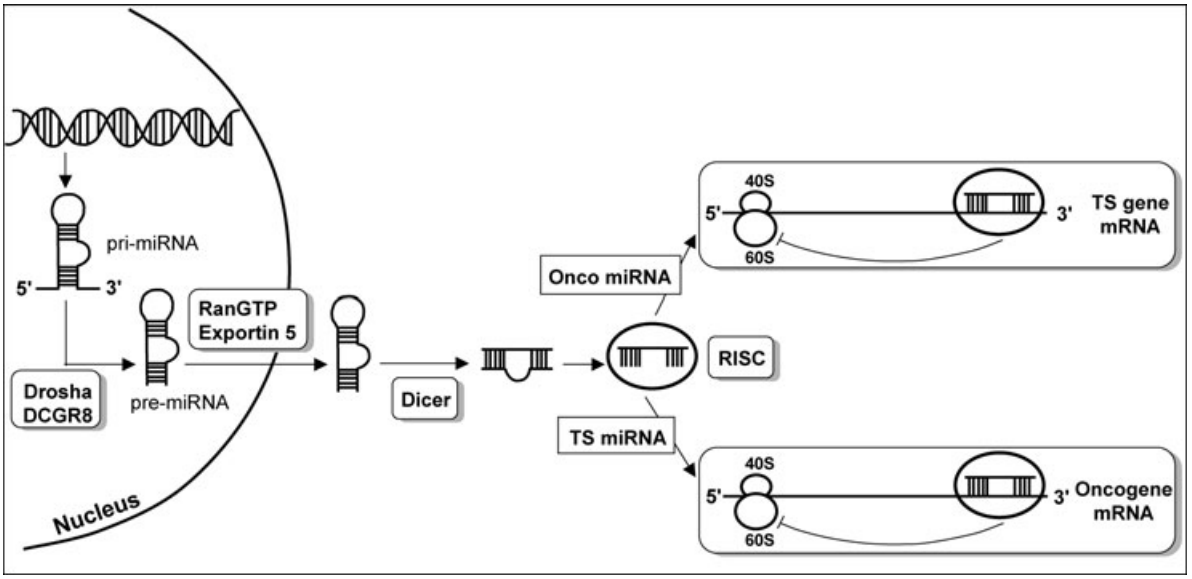

Fig. 1 Mechanism of action of TS and onco miRNAs. MiRNAs are transcribed in the nucleus and the primary transcripts (pri-miRNAs) are processed by the nuclear RNase III Drosha in cooperation with DCGR8. The resulting pre-miRNAs are exported to the cytoplasm by Exportin 5/ RanGTP and further processed into double strand intermediates by the cytosolic RNase III Dicer. Mature miRNAs are then loaded into the RNA-induced silencing complex (RISC), where they imperfectly pair with mRNA targets (typically at the 3′UTR, in the seed match sequence) to direct post-transcriptional repression by translation inhibition or mRNA destabilization. MiRNAs behave as oncogenes when they target tumour suppressor genes; conversely they can behave as TS genes when they target oncogenes. In some cases miRNAs may have a double role as TS or oncogene depending on the type of cancer.

for aging [7, 8]. One of the commonest points of view of aging is that telomere shortening and accumulation of DNA damage occurs during the lifespan of the organism in stem cells as well as in more committed cells. Thus aging could result from the exhaustion of the regenerative potential of stem cells, although at present it is still a matter of debate whether senescence is the only mechanism responsible for stem cell pool exhaustion [7].

Although diverse stimuli can induce senescence, they appear to converge on either or both of the two main pathways that establish and maintain senescence growth arrest. These pathways are governed by the tumour suppressor proteins, p53 and Retinoblastoma (RB) [9–11]. One of the main upstream controllers of the p53 and RB pathways is the *INK4a/ARF* locus. This locus encodes, by alternate reading frames, two different proteins p19ARF (mouse) or p14ARF (human) and p16INK4a [12], which activate, respectively, p53 and RB [13]. As expected this tumour suppressor locus is frequently found deleted or rearranged in various types of cancer such as glioblastoma, melanoma, pancreatic adenocarcinoma, non-small cell lung cancer, bladder carcinoma, oropharyngeal cancer [12] and primary lymphoma [14].

MicroRNAs are implicated in tumourigenesis and senescence

MicroRNAs (miRNAs) are non coding short 22 nt RNA molecules which have recently come to stage as important players in basic cellular functions such as cell proliferation, differentiation apoptosis and senescence [15, 16]. Interestingly, increasing evidence indicates that many disease states such as cardiovascular, neurodegenerative, liver and kidney diseases [16] as well as cancer [17] occur or are sustained by miRNA disregulation. Expression of miRNAs changes markedly in tumours [18] and an increasing body of evidence indicates that they can behave either as tumour suppressor genes (TS miRNAs) or as oncogenes (onco miRNAs) [19–21], although it is becoming clearer that some miRNAs have a double role (TS and onco miRNAs) according to the cellular context [22]. MiRNAs are negative regulators of gene expression by imperfectly pairing to sequences (named seed match) in the 3′UTR of the target mRNA and inhibiting its translation [23]: thus miRNAs may behave as oncogenes when they inhibit tumour suppressor genes, while their tumour suppressor activity is due to the inhibition of oncogenes (Fig. 1). TS miRNAs, identified as they were markedly under-expressed in tumours, have been shown to play a role both in senescence and in apoptosis (the two main tumour suppressor mechanisms). A list of the most common TS miRNAs as well as of miRNAs with a double behaviour (TS and onco miRNAs) and their relative targets is given in Table 1. It is evident that while few TS miRNAs have been shown to exert a pro-senescence activity, others target well known anti-senescence genes suggesting a possible role in senescence. One of the first discovered TS miRNAs was let-7, down-regulated in various solid tumours [19, 24]. The proto-oncogene RAS was the first validated target of let-7 [25], but recently let-7 has also been shown to have a role in senescence and

Table 1 MiRNAs implicated in tumourigenesis with a role in senescence

miRNA	Relevant targets	Function in cancer	Involvement in senescence	References
Let-7 family	RAS, cMYC, HMGA2, CCND1, CDC25a, CDC34, CDK6	Tumour suppressor	+	[25, 26, 66]
miR-34 family	SIRT1, cMYC, E2F3, HMGA2, BCL2, CCND1, CCNE2, CDK4/6	Tumour suppressor	+	[27–29]
miR-26a	EZH2, CCNE2, CCND2	Tumour suppressor	+?	[31, 32]
miR-143, miR-145	cMYC, ERK5, kRAS	Tumour suppressor	+?	[34, 67, 68]
miR-101	EZH2, COX2, MCL1	Tumour suppressor	+?	[69–71]
miR-29 family	DNMT3A/B, CDC42, BCL2, MCL1	Tumour suppressor	–	[35–37]
	TTP	Oncogene	–	[72]
miR-125a/b	E2F3, ERBB2/3	Tumour suppressor	–	[39, 73]
	p53	Oncogene	–	[56]
miR-17	E2F1, AIB1	Tumour suppressor	+?	[38]
	E2F1	Oncogene	–	[51]

AIB1, amplified in breast cancer 1; CCND1, cyclin D1; CCND2, cyclin D2; CCNE2, cyclin E2; DNMT3A, DNA methyltransferase 3A; DNMT3B, DNA methyltransferase 3B; ERBB2, v-erb-b2 erythroblastic leukaemia viral oncogene 2; ERBB3, v-erb-b2 erythroblastic leukaemia viral oncogene 3; ERK5 extracellular signal-regulated kinase-5; HMGA2, high-mobility group AT-hook 2; MCL1, myeloid cell leukaemia sequence 1 and TTP, tristetraprolin. Targets in bold are significant for senescence.
(+), miRNAs which induce senescence; (+?), miRNAs which regulate senescence associated genes and (−), miRNAs not associated to senescence.

aging by targeting a negative regulator of the *INK4a*/*ARF* locus, the so-called high-mobility group AT-hook 2 (HMGA2) protein [26].

Recently, attention has been focused on miRNAs regulated by the tumour suppressor gene p53; these studies have shed new light on how this master gene regulator utilizes miRNAs to induce senescence. One of the most studied p53 responsive miRNA is miRNA-34a (miR-34a), which in human colon cancer cells induces growth arrest accompanied by morphological and biochemical changes characteristic of senescence (such as enlarged cellular size and β-galactosidase$^+$ cells) [27]. Sirtuin 1 (SIRT1) [28] and the transcription factor E2F3 [27] are some of the targets involved in miR-34 induced senescence. Interestingly miR-34a also targets the myelocytomatosis oncogene (cMYC) [29], which in turn represses various TS miRNAs [30] among which miR-26a successfully used in replacement therapy in liver cancer [31]. As miR-26a specifically targets the methyl transferase enhancer of zeste homolog 2 (EZH2) [32], a known repressor of the *INK4a*/*ARF* locus [33], it could play a role in senescence by activating the p53 and RB pathways. Another p53 controlled TS miRNA with pro-senescence activity, is miR-145, which, by targeting cMYC [34], could induce miR-26a up-regulation, with consequent activation of the *INK4*/*ARF* locus, p53 and RB. The list ends with the few so far identified miRNAs (miR-29a, miR-125a/b and miR-17–5p) with a dual function of TS and onco miRNAs. It is interesting to point out that while miR-29a exerts its TS activity by down-regulating genes not immediately related to senescence [35–37], both miR-17–5p [38] and miR-125b [39] have been shown to block cancer cell proliferation *via* E2Fs inhibition, which is known to induce senescence [40].

The examples shown in the table reinforce the concept that miRNAs affect the expression of multiple genes, many of which modulate directly or indirectly the p53 and RB pathways; moreover the cellular context is fundamental in determining their final effect. We believe that the pro-senescence role exerted by miR-20a [41] and miR-290 [42] in MEF is a proof of these principles and highlights the importance of understanding the mechanisms of action of miRNAs in different cellular contexts.

MiR-20a and miR-290 induce senescence in MEF

As previously mentioned the *INK4a*/*ARF* locus is the master controller of p53 and RB pathways and as a consequence it regulates senescence and apoptosis. In turn this locus is tightly controlled by a series of activators and repressors, in order to prevent tumourigenesis [33]. As expected, many of the genes which suppress this locus have oncogenic activity. In MEF one of them is the leukaemia/lymphoma related factor (LRF) which, by specifically repressing the transcription of the tumour suppressor p19ARF, bypasses the senescence response elicited by transfection of single oncogenes [43]. *In silico* analysis showed that LRF 3′UTR contains target sites for at least seven miRNA families [44] among which miR-20a, part of the miR-17–92 cluster, involved in tumourigenesis. The cluster behaves as an oncogene in different

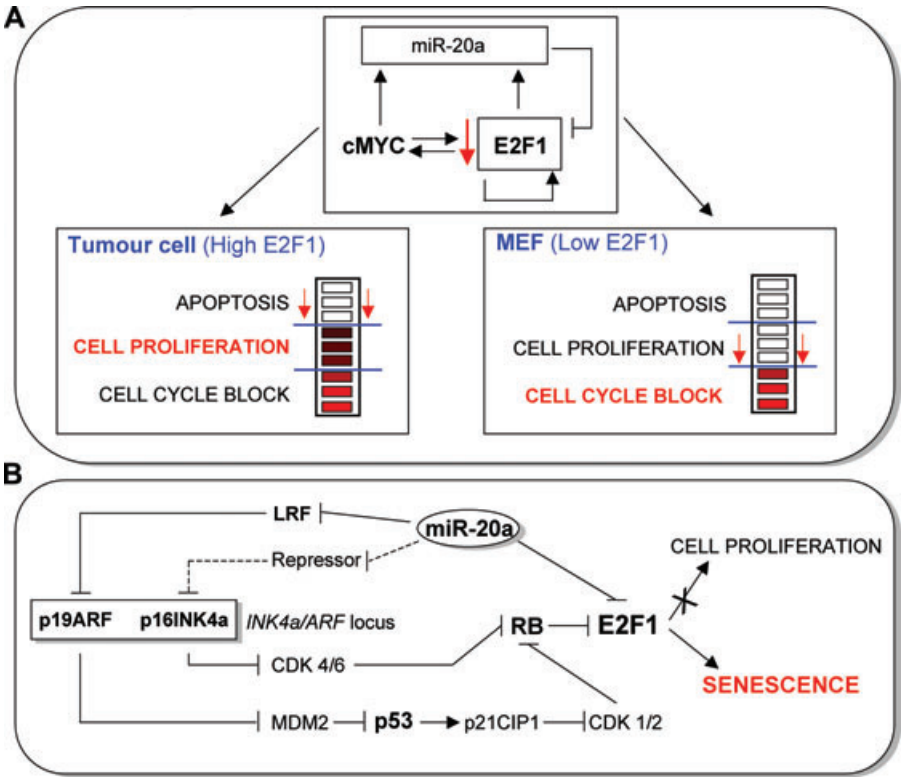

Fig. 2 MiR-20: a multifaceted miRNA which affects multiple pathways. (**A**) MiR-17–92 cluster is part of a self-regulating circuit: schematically cMYC binds the promoter of the cluster as well as that of E2F1 increasing their transcription; in turn E2F1 induces the cluster transcription. MiR-20a, a member of the cluster, directly targets E2F1 in order to control its level. In tumour cells, where E2F1 level is high, miR-20a increases the oncogenic power of cMYC by keeping E2F1 level below the pro-apoptotic threshold (left side). This concept is schematically visualized as a bar which represents the full range of cellular E2F1 level variation; the red rectangles within the bar represent the actual E2F1 quantities which determine the final biological outcome (cell proliferation in the case of tumour cells). On the contrary, in MEF, where E2F1 level is limiting, miR-20a induces cell cycle block and senescence by down-regulating E2F1 below the cell proliferation threshold (right side). Blue lines represent thresholds between the different biological outcome (apoptosis, proliferation and cell cycle block). (**B**) miR-20a induces senescence by affecting multiple pathways: it down-regulates LRF thereby stabilizing p53 *via* p19ARF activation, it down-regulates directly E2F1 and indirectly up-regulates p16INK4a. Dashed lines indicate hypothetical pathways.

types of tumours [45, 46] among which lymphomas [47], while it is deleted in other types of cancers [48–50] suggesting a tumour suppressor role. The interaction between miR-20a and LRF 3′UTR was experimentally validated and, as expected, miR-20a overexpression in MEF provoked LRF down-regulation with consequent p19ARF increase and senescence induction [41]. However, the power of miR-20a to induce senescence goes beyond LRF down-regulation, as it is a stronger senescence inducer than a short interfering RNA specific for LRF (siLRF). Indeed miR-20a overexpression in MEF also induced a marked down-regulation of E2F1 along with LRF, so it is likely that the combined depletion of these two factors contributes to senescence [41].

Double role of miR-20a as oncogene and pro-senescence gene

The pro-senescence role of miR-20a [41] appears to be in contrast with its oncogenic role [47]; however a more subtle interpretation of the data highlights a particular aspect of miRNA properties discussed above: *i.e.* the cellular context may be decisive for the final effect of miRNAs. In tumour cells the miR-17–92 cluster increases the oncogenic power of cMYC in a self-regulating circuit whereby cMYC binds the promoter of the miR-17–92 cluster and increases its transcription [51]. In turn two miRNAs of the cluster, miR-17–5p and miR-20a (belonging to the same seed family), target E2F1, which is able to activate both the cluster and cMYC [52]. In other words, cMYC, while directly increasing the transcription of E2F1, indirectly decreases its translation by inducing miR-17–5p and miR-20a. In this way, cMYC uses the miR-17 seed family to maintain E2F1 protein levels below the pro-apoptotic threshold allowing the proliferative signal to prevail (Fig. 2A, left side) [53]. Conversely in MEF, where E2F1 levels is limiting, its down-regulation by miR-20a contributes to senescence (Fig. 2A, right side) in agreement with the idea that miRNAs might have opposite effects in different cells. In this regard it would be interesting to test miR-20a in tumours where inhibition of E2F1 transcriptional activity has been found to block tumour growth [27, 54].

MiR-20a activates the *INK4a/ARF* locus

Intriguingly, in MEF, miR-20a not only increased p19ARF (*via* LRF down-regulation), but also p16INK4a [41], the other protein encoded by the *INK4/ARF* locus, by unknown mechanism (Fig. 2B).

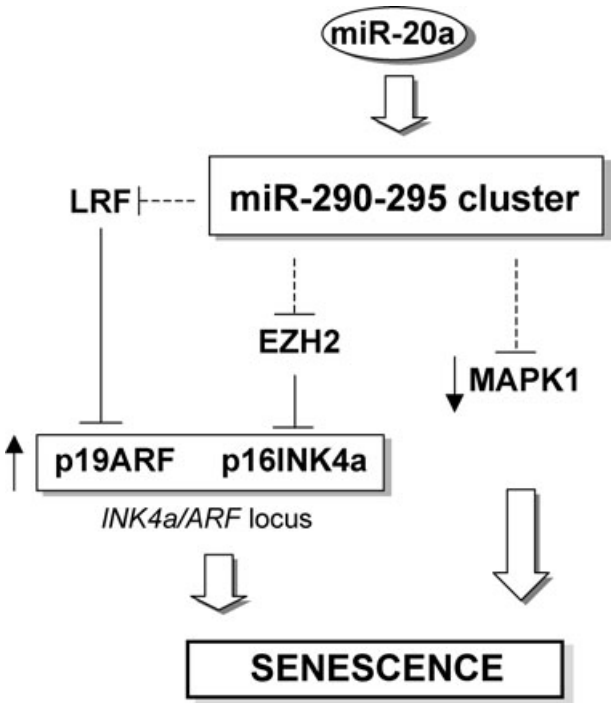

Fig. 3 MiR-290–295 cluster is causatively involved in MEF senescence. MiR-290–295 cluster induces senescence by activating the *INK4a/ARF* locus. Possible mechanisms are: (*i*) LRF down-regulation with activation of p19ARF and p53 and (*ii*) p16INK4a up-regulation by EZH2 down-regulation. Other candidate targets are members of the MAPK family, among which MAPK1/ERK1, known to activate cell proliferation. The induction of p16INK4a by miR-20a (see Fig. 2C) could be mediated by miR-290–295 cluster.

These results indicated that mRNAs other than those of LRF and E2F1 are affected by miR-20a, in keeping with the idea that miRNAs regulate the expression of multiple genes, so their final biological effect depends on the sum of the affected targets [55]. In this regard it is worth mentioning that miR-100 and miR-125b, which directly down-regulate LRF, did not induce senescence in MEF, but rather increased cell proliferation [42]. These unexpected results may find an explanation in a recently published paper, showing that one of the targets of miR-125b is p53 [56], which has strong anti proliferative and pro-senescence properties in MEF [57].

MiR-290–295 cluster is causatively connected with culture-induced senescence in MEF

In accordance with the idea that miRNAs control pathways, we found that senescence, induced by different stimuli (culture, siLRF or miR-20a) is accompanied by up-regulation of a specific set of miRNAs [42]. Singularly, miR-290 (belonging to the miR-290–295 cluster) was the most overexpressed in the three conditions [42]. The time course of miR-290 up-regulation during culture-induced senescence shows that this miRNA, along with other members of the cluster (specifically miR-291–3p, miR-292–3p and miR-295) is expressed at low levels in early passages MEF but steadily increases during cell propagation in culture with a time course which paralleled LRF down-regulation [42]. LRF down-regulation is due to post-transcriptional silencing as the mRNA levels remain constant, suggesting that modulation by miRNAs could play a role. Interestingly, while miR-20a is unlikely to be responsible for LRF down-regulation as it diminishes along with passages (Verduci *et al.*, *JBC*, under revision), miR-292–3p, which increases during senescence [42], could potentially target LRF [44].

The *INK4a/ARF* locus is implicated in miR-290 induced senescence

A recurrent result found in culture-induced senescent MEF is the consistent association between miR-290 up-regulation and the increased expression of the *INK4a/ARF* locus (p19ARF and mainly p16INK4a) [42]. It has been shown that the increase of p16INK4a and to a lesser extent that of p19ARF observed during senescence in primary fibroblast (including MEF) is due to down-regulation of EZH2, part of the PRC2 complex which inhibits the *INK4a/ARF* locus [58]. *In silico* analysis and preliminary experiments utilizing a gene reporter assay indicate that the 3′UTR of murine EZH2 is a target of miR-290 (Rainaldi G., unpublished data). Future studies will establish whether EZH2 down-regulation during MEF senescence is causatively connected to miR-290 up-regulation; if this were the case p16INK4a increase observed after miR-20a overexpression [41] could be miR-290 dependent (Fig. 3).

Finally, the mechanisms by which miR-290 induces senescence may be multiple, indeed other interesting predicted targets of miR-290 are various members of the mitogen-activated protein kinase (MAPK) family, among which MAPK1/ERK1, known to activate cell proliferation [59]. Thus it is possible that miR-290 drives cells towards senescence by the combined down-regulation of cell proliferation signalling pathways (MAPK), and up-regulation of cell cycle inhibitors such as p16INK4a (*via* EZH2 inhibition) (Fig. 3).

The double life of miR-20a and miR-290 in stem cells and MEF

MiR-290–295 cluster is expressed in stem as well as in senescent cells

MiR-20a [41] and miR-290 [42] belong to two clusters (respectively, miR-17–92 and miR-290–295) which represent the

majority of miRNAs expressed in embryonic stem (ES) cells [60]. MiR-290–295 cluster in particular is important not only to maintain pluripotency in ES cells [60, 61], but also to increase the efficiency of MEF reprogramming to induced pluripotent stem (iPS) cells by the transcription factors Oct4, Sox2 and Klf4 [62]. This work [62] demonstrated that in early passages MEF the miR-290–295 cluster is silenced by methylation of histone 3 lysine 27 (H3K27) on the promoter and can be re-expressed after transfection with the three above mentioned transcription factors and cMYC. The re-expression takes place late in the reprogramming process, suggesting that miR-290–295 cluster is downstream of cMYC and requires epigenetic remodelling before being expressed. We clearly showed that the whole miR-290–295 cluster is up-regulated in MEF during serial passages in culture, the maximum up-regulation being when cells reach senescence [42]. These data are not in contrast with the work of Judson *et al.* [62] rather suggesting that during senescence a remodelling of the chromatin takes place with removal of transcriptional silencing of the cluster, permitting its transcription. As during MEF senescence two chromatin modifiers, the trimethylase EZH2 [58] as well as the deacetylase recruiter LRF [42] are down-regulated, it will be interesting to explore whether they are causally connected to miR-290–295 cluster de-repression.

The cellular context influences the final biological effect of miR-20a and miR-290

Both miR-20a and miR-290 belong to the also called ES-cell-specific cell-cycle-regulating (ESCC) miRNAs, required to regulate G1-S transition and promote rapid cell proliferation [61]; it is then singular that the both miRNAs exert a pro-senescence role in MEF [41, 42]. We suggest that the opposite effects of miR-20a [41] and miR-290 [42] in MEF and ES cells may depend on the presence of (1) different miRNAs repertoire as well as (2) target/s level/s present in the two cellular contexts.

(1) As previously mentioned, transfection of ESCC miRNAs in MEF together with ES specific transcription factors induce iPS cells [62]; in a subsequent paper [63] the same authors found that depletion of let-7, normally expressed at high levels in MEF [60], enhances MEF reprogramming. They propose that let-7 family and miR-290–295 cluster have opposing effects acting in self-reinforcing loop to maintain the ES cell self-renewal *versus* differentiated state; according to this model let-7 should never be co-expressed with the miR-290–295 cluster. In this regard it would be interesting to verify whether MEF undergoing senescence continue to express high levels of let-7 and in this case whether the concomitant expression of miR-290 and let-7 has a causative role in senescence.

(2) Computational analysis has predicted that in ES cells the two miRNA clusters (miR-17–92 and miR-290–295) operate by a series of so-called type I circuits [64], where both miRNAs and putative targets are positively correlated; the hypothetical model is that miRNAs allow the translation of the target genes which are transcribed above critical threshold [64]. In this way the role of the miRNAs is to fine tune the target expression, avoiding unwanted excess: examples of this type of circuit are represented by miR-17–92 cluster co-expressed with high levels of E2F1, and by miR-290–295 cluster co-expressed with high levels of EZH2 [64, 65]. It is worth noting that the miR-17–92/E2F1 connection in ES cells, closely resembles that described in tumour cells (Fig. 2A, left side), establishing a potential link between ES and cancer cells [65]. We suggest that in MEF, miR-20a and miR-290 operate in so-called type 2 circuits, where miRNAs and their targets are negatively correlated [64]; in particular miR-20a and miR-290 up-regulation takes place in a situation where the respective targets (E2F1 and EZH2) are limiting so the final effect is down-regulation of the proteins below a critical threshold, with consequent cell proliferation inhibition and senescence induction.

Concluding remarks

In conclusion the mechanism of action of miR-20a [41] and miR-290 [42] in MEF reinforces the concept that miRNAs affect pathways rather than single genes, so that even small perturbation of miRNA levels may have a significant impact on cell fate. Moreover, the final biological effects of miRNAs strongly depends on the repertoire of miRNAs, mRNA targets, and their level, expressed in cells, so the same miRNA may have opposite roles (TS miRNA or onco miRNA) in different cellular context. As recent studies have highlighted the therapeutic properties of miRNAs against cancer, significant work remains to be done in order to determine, as accurately as possible, their potential tumour suppressor activity in diverse genetic settings.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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