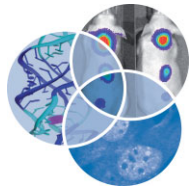
Advanced Review



Mirtrons, an emerging class of atypical miRNA

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Post-transcriptional gene silencing (PTGS) via RNA interference (RNAi) is a vital gene regulatory mechanism for fine-tuning gene expression. RNAi effectors termed microRNAs (miRNAs) are implicated in various aspects of animal development and normal physiological function, while dysregulation has been linked to several pathologies. Several atypical miRNA biogenesis pathways have been identified, yet in most cases the reasons for their emergence remain unclear. One of these atypical pathways is the mirtron pathway, where short introns are excised by splicing to generate intermediates of the RNAi pathway, with no cleavage by the microprocessor. Closely related pathways involving tailed-mirtron and simtron biogenesis have also been described. There is extensive evidence that mirtrons function as miRNAs, and while some are evolutionarily conserved across similar species, others appear to have emerged relatively recently. In addition, through exploitation of the potent and sequence-specific silencing capabilities of RNAi, synthetic mirtrons may have potential for overcoming certain therapeutic challenges.

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INTRODUCTION

Post-transcriptional gene silencing (PTGS) via RNA interference (RNAi) has attracted great interest as a gene regulatory mechanism in recent years because of its potential to fine-tune gene expression and increase complexity arising from the genome. Through endogenous genome-encoded effectors termed microRNAs (miRNAs), the process of RNAi is implicated in various aspects of development and normal physiology, while dysregulation has been linked to several pathologies ranging from cancer to neurodegenerative disease.[1–6] The exploitation of the sequence-specific silencing activity of RNAi has potential as a promising antisense therapeutic strategy,[6] and the first clinical trials have already been reported.[7,8] While research over the last 14 years has identified RNAi processing pathways that appear applicable to most miRNAs across mammals and invertebrates,[9–13] in the past few years several atypical miRNA biogenesis pathways have been identified.[14–29] In most cases the reasons for their emergence remain unclear. In this review, we look in detail at one of these atypical pathways, the mirtron pathway, which utilizes splicing rather than microprocessor activity to generate intermediates of the RNAi pathway.

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MICRO-RNA PATHWAYS

Gene Silencing by the Canonical miRNA Pathway

The canonical miRNA pathway involves step-wise processing of long genome-encoded primary-miRNA (pri-miRNA) transcripts into short double-stranded miRNA duplexes of ~19-21nt with 2-nt 3′ overhangs, sometimes known as short-interfering RNAs (siRNAs), which act as substrates for the gene-silencing machine[30,31] (Figure 1(a)). In the first step, a pri-miRNA is transcribed either from an intergenic region off its own separate promoter, or alternatively within an intron of a protein coding gene. Following transcription, the pri-miRNA is processed by a microprocessor complex containing the nuclease

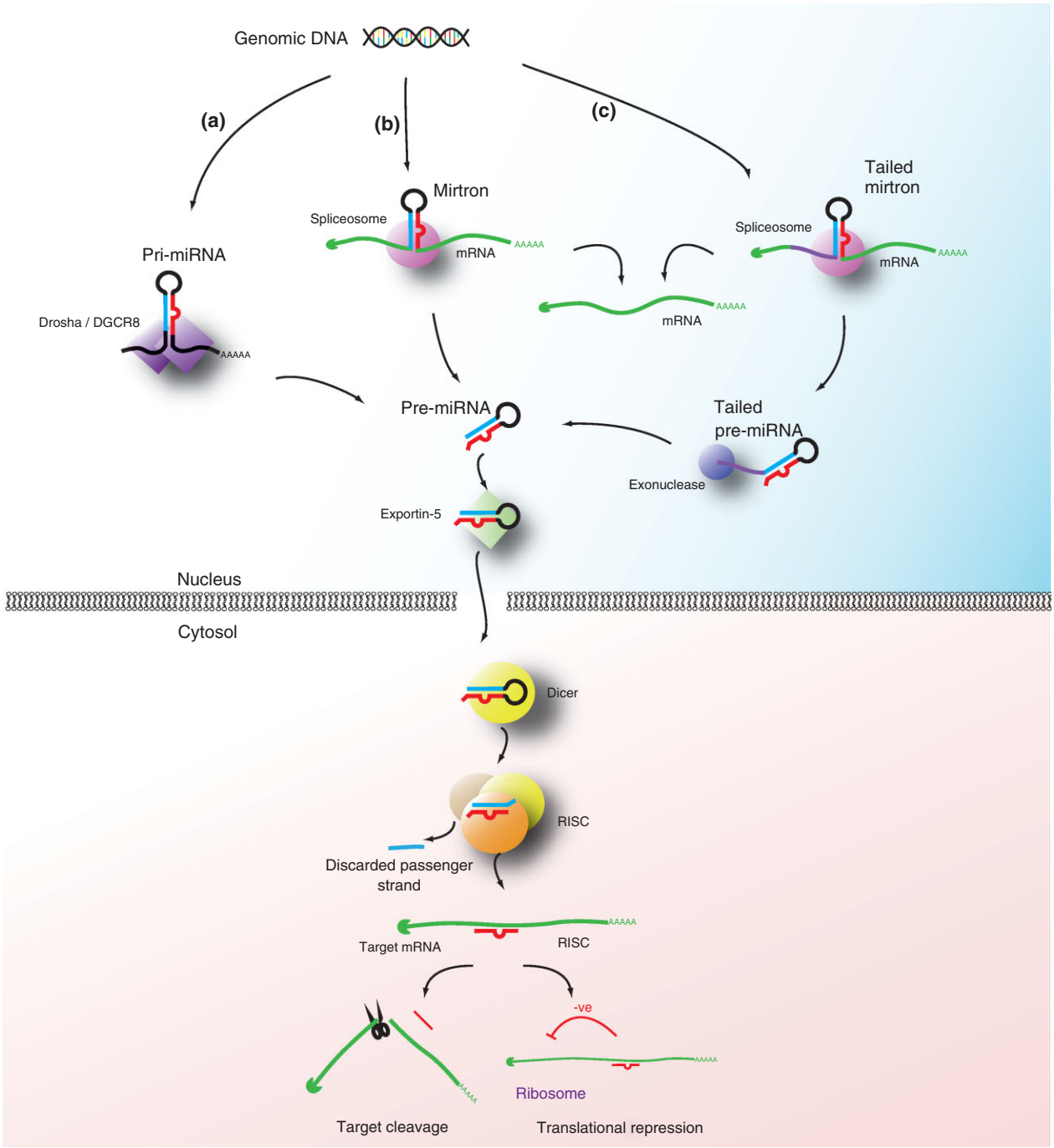

FIGURE 1 | Canonical miRNA and mirtron pathways. (a) In the canonical mammalian miRNA pathway, pri-miRNA transcripts are processed by the microprocessor complex (Drosha/DGCR8) into pre-miRNA hairpins that are recognized by Exportin-5 for nuclear export. Further processing by Dicer, recruitment into RISC, and strand selection result in a mature antisense species capable of directing translational repression of imperfectly matched targets or target cleavage of perfectly matched targets. (b) In the mirtron pathway, pre-miRNA hairpins are produced directly from introns via splicing of pre-mRNAs, whereby functional mature mRNAs are also produced. The hairpins are then thought to join the canonical pathway at the stage of nuclear export, not requiring any processing by Drosha. (c) Tailed mirtrons derive from slightly larger introns, and are spliced from pre-mRNA, but a single tail remains on one strand (here the 5′ strand), which is digested by an exonuclease. Again these hairpins then join the canonical pathway for nuclear export.

Drosha and its cofactor, DiGeorge syndrome critical region 8 (DGCR8), known as Pasha in invertebrates. Substrate recognition by the microprocessor requires an extended stem-loop importantly flanked by single-stranded RNA.[9] This yields a shortened precursor-miRNA (pre-miRNA) bearing just the stem-loop hairpin with a 2-nt overhang at the 3′ end, which acts as a recognition signal for Exportin-5.[10] Subsequent nuclear export of the pre-miRNA by Exportin-5 and processing by the nuclease Dicer, known as Dicer-1 in *Drosophila*, produces the double-stranded miRNA. These miRNAs associate with an Argonaute (AGO)-containing protein complex to assemble the RNA-induced silencing complex (RISC) responsible for gene silencing.[11] In mammals there are four AGO proteins (AGO1-4), while in *Drosophila* and *Caenorhabditis elegans* there are two, dAGO1/2 and ALG1/2, respectively. One 'antisense' strand of the miRNA duplex, known as the mature miRNA, is preferentially retained based on thermodynamic stability while the other is discarded,[12] and then the activated RISC scans mRNA transcripts for sequence complementarity. If the antisense RNA and mRNAs have significant yet incomplete base pairing, as typically seen with these endogenous mature miRNAs and their 3′ untranslated region (3′ UTR) targets, translation is repressed.[32] It appears that all AGO proteins are involved in this effect in mammals, both ALG1/2 are involved in *C. elegans,* while dAGO1 is responsible in *Drosophila*. In contrast, where there is a complete base pairing, as commonly utilized in synthetic RNAi approaches which exploit miRNA pathway, the mRNA transcript is degraded in a manner which absolutely requires AGO2 in mammals.[33]

In mammals and invertebrates, it is well established that miRNAs tend to regulate the expression of mRNAs containing multiple complementary target sites within their 3′UTRs. However, recent findings have suggested that miRNAs may also target 5′UTRs,[34] ORFs,[35] and promoter regions.[36] A 'seed' region of the mature miRNA has been suggested which corresponds to positions 2–9 of the antisense sequence, and this has been demonstrated to have a disproportionate role in mRNA target binding.[37,38] With few exceptions it appears that near-complete complementarity between the seed region and a 3′UTR target site is necessary for the initiation of RNAi, whereas pairing in the more distal positions of the mature miRNA seems to regulate the rate of RISC catalysis.[38] These rules have allowed the development of several target prediction algorithms, which have importantly indicated that each individual miRNA often has the potential to repress translation of many mRNA targets.[39,40] Indeed, this has been confirmed with more recent high-throughput approaches that have identified miRNA target networks.[41,42] Collectively it implies that the human mature miRNAs now identified (more than 1500) may post-transcriptionally regulate ∼30% of the human mRNA transcriptome. Further, over 20,000 mature miRNAs have been reported across 168 different organisms, suggesting that the miRNA pathway is a vast post-transcriptional regulation network with important roles in both health and disease across many levels of evolution. Grasping an understanding of the many aspects of this pathway will therefore be crucial for furthering knowledge of basic biology and in the development of new therapeutics.

Atypical miRNA Biogenesis

While the canonical pathway has been described, recent findings have led to an appreciation that natural variations in the biogenesis of miRNA exist, adding further complexity to RNAi regulation within the cells. Several Drosha-independent mechanisms of biogenesis have been reported. These include the topic of this review, mirtrons, which are a novel class of splicing-dependent miRNAs originally identified in *Drosophila melanogaster* and *C. elegans*,[14,15] and since confirmed in mammals.[16–18] Biogenesis involves the removal of short introns with hairpin-forming potential to form pre-miRNA-like hairpins that bypass Drosha/DGCR8 processing (Figures 1(b) and 2(a)). The closely related tailed mirtrons are processed by both splicing and exonuclease digestion[16,19] (Figures 1(c), 2(b), and (c)). Drosha-independent routes also exist which do not involve splicing. For example, two miRNAs, miR-320 and miR-484, appear to be transcribed as short pre-miRNA-like hairpins devoid of Drosha/DGCR8 recognition motifs and are instead processed directly by Dicer[17] (Figure 2(d)). Alternatively, miRNAs can be processed by unknown Drosha-independent mechanisms from small nucleolar RNAs (snoRNAs) bearing pre-miRNA-like hairpin structures which form Dicer substrates[20] (Figure 2(e)). Interestingly, these snoRNAs are still active in conjunction with ribonucleproteins suggesting that not all copies are processed into mature miRNAs. Transfer RNA (tRNA)-like structures can also derive mature miRNAs, originating from both endogenous mammalian RNAs[17,21] and also the murine γ-herpesvirus 68 (MHV68).[22] Again these are Drosha-independent, yet slight variation in the biogenesis is seen between the two. In mammalian examples the miRNA appears to be processed from the tRNA structure exclusively by Dicer

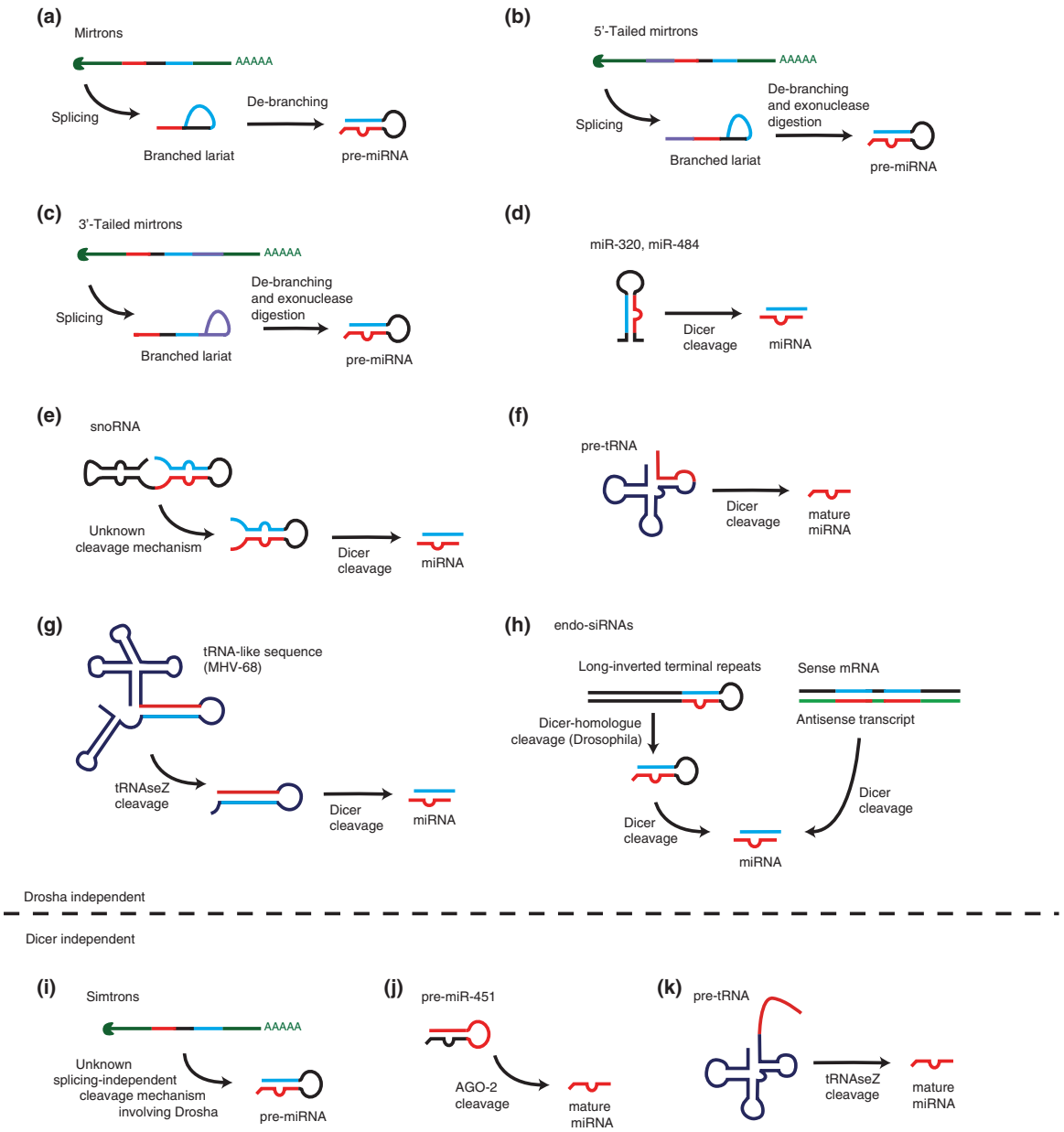

FIGURE 2 | Atypical biogenesis pathways for miRNA generation. (a) The mirtron pathway involves splicing of short introns with hairpin-forming potential. Following debranching of the branched lariat intermediate of the splicing pathway, sequence homology between 5′ and 3′ ends of the intron allows pre-miRNA-like hairpins to form. (b, c) Tailed mirtrons are very similar to mirtrons, arising from short introns with hairpin-forming potential. However, following debranching, the pre-miRNA-like hairpin has a single-stranded tail on either the 5′ or 3′ end which requires exonucleolytic cleavage by a Drosha-independent mechanism. (d) miR-320 and miR-484 are pri-miRNA sequences devoid of Drosha recognition characteristics. Dicer processes the hairpins directly to release mature miRNAs. (e) Some miRNA map to snoRNAs containing secondary structures mimicking Dicer substrates. Following initial processing by a Drosha-independent mechanism, hairpin structures are processed by Dicer into mature miRNAs. (f) tRNA-like secondary structures can be processed by Dicer to release short RNA species from their 3′ ends in mammals. (g) miRNAs encoded by the MHV68 virus derive from complex secondary structures incorporating pol-III promoters and which are analogous to tRNAs. In initial processing tRNAaseZ cleave to release a hairpin sequence that is recognized by Dicer. (h) Endogenous siRNAs can derive from long inverted repeat sequences that are cleaved by a homolog of Dicer in Drosophila to shorter hairpins recognized by Dicer. Alternatively, mRNA transcripts can anneal to complementary antisense transcripts and form dsRNA duplexes that are recognized and processed by Dicer. A number of pathways are also known which are independent of Dicer. (i) Some mirtron-like miRNAs may be processed via the simtron route, requiring Drosha but not splicing, DGCR8, or Dicer. (j) Pri-miR-451 is processed by Drosha into a hairpin shorter than canonical Dicer substrates, and the mature miRNA enters the hairpin loop. AGO2 has been found to mediate processing of the pre-miRNA into the mature sequence. (k) In addition to Dicer-dependent cleavage of tRNAs, tRNAseZ can also cleave 3′ sequences of precursor tRNAs to release small RNAs that can enter gene-silencing pathways.

(Figure 2(f)), whereas MHV68 miRNAs are processed by tRNaseZ prior to further processing by Dicer (Figure 2(g)). Lastly, a group of 21-22-nt endogenous-siRNAs (endo-siRNAs) arise from multiple origins (Figure 2(h)). For example, a long antisense transcript (>100-nt), produced from an antisense promoter either in the same gene or a homolog, can hybridize with an mRNA and be processed by unknown mechanisms involving Dicer into multiple small RNAs capable of RNAi. Inverted repeat hairpins, much longer than canonical Drosha substrates, can also be processed to produce active endo-siRNAs.[23–25]

Dicer-independent miRNAs have also been reported. Some mirtron-like miRNAs may be processed via the simtron route, which involves Drosha but not splicing, DGCR8, or Dicer[26] (Figure 2(i)). One miRNA, miR-451, is first processed by Drosha before AGO2 recognizes and cleaves the resulting pre-miRNA within the loop region to release the mature miRNA[27–29] (Figure 2(j)). A collection of putative miRNAs also appear to derive from the 3′ end of tRNAs[21,43] (Figure 2k). Specifically, tRNAse Z has been shown to remove short sequences of comparable length and chemistry to canonically processed miRNAs, suggesting that these may also be functional in gene silencing pathways. However, this awaits full confirmation.

While the functional significance of these atypical miRNA biogenesis pathways remains unknown in most cases, in recent years there has been increasing interest in the mirtron pathway that creates approximately 1%–2% of all the human miRNAs. In the remainder of this review we focus on this pathway and also its close relatives, namely tailed mirtrons and simtrons.

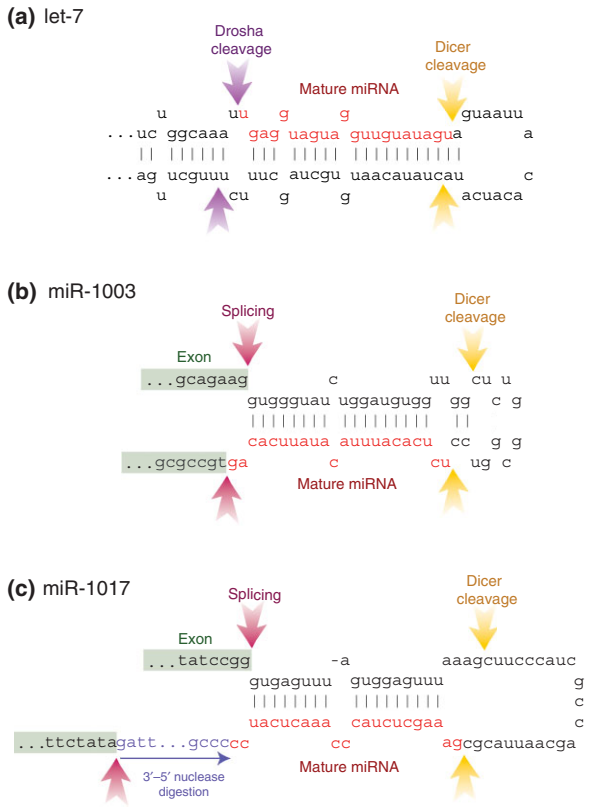

FIGURE 3 | Examples of canonical miRNAs, mirtrons, and tailed mirtrons from *Drosophila melanogaster*. (a) let-7, a canonical miRNA. The primary transcript contains an extended hairpin which is first cleaved by Drosha to produce the pre-miRNA. The loop region is then removed by Dicer and the mature miRNA strand is incorporated into RISC. (b) miR-1003, a mirtron: a miRNA produced from an intron without Drosha cleavage. The primary transcript is spliced, forming a functional mRNA and a pre-miRNA hairpin which is cleaved by Dicer as in (a). (c) miR-1017, a tailed mirtron. The primary transcript is spliced as in (b), except that the pre-miRNA hairpin produced contains a tail of around 100 nucleotides on the 3′ arm. This is digested by a 3′-5′ exonuclease (the RNA exosome in *Drosophila*).[19]

MIRTRON BIOLOGY

Identification and Biogenesis of Mirtrons in Invertebrates

The first mirtrons were identified by Ruby et al. from the examination of *D. melanogaster* short RNA sequencing data.[14] Specifically a pair of short complementary RNAs were identified which originated from either end of a short (56-nt) intron of comparable length to *D. melanogaster* pre-miRNAs. The pairing of the intron ends gave a 2-nt 3′ overhang, the canonical signal for Exportin-5 nuclear export,[10] and pairing of the two short RNAs had 2-nt 3′ overhangs, the canonical result of Dicer processing. This further hinted toward a function of this intron as a miRNA. Given that the complementary pairing forms a hairpin structure that does not continue beyond the canonical splice sites, it was proposed that this miRNA must bypass the canonical microprocessor step of Drosha and Pasha activity, which appears to involve recognition of a double-stranded stem region extending from the ends of the mature miRNA and flanked by single-stranded RNA regions[9] ((Figure 3(a)). This new miRNA was annotated miR-1003 ((Figure 3(b)). Searches for other small RNAs with similar characteristics identified 13 others and these short intron pre-miRNAs were termed mirtrons (Table 1). In addition, one previously annotated *C. elegans* miRNA was also classified as a mirtron, miR-62, while three more candidate mirtrons were identified amongst *C. elegans* small RNA libraries.

It was shown that the processing of miR-1003 is dependent on splicing. Firstly, mutating either the 5′ or

TABLE 1 | Mirtrons That Have Been Confidently Identified

Organism Type	Species	Mirtrons	Tailed Mirtrons
Invertebrates	*C. elegans*	miR-62[14]	
	Drosophila	miR-1003, 1004, 1010[14,15]	miR-1017[19]
Plants	Rice	miR-1429.2,[44,45] [17, unnamed][45]	
	Arabidopsis	[5, unnamed][45]	
Mammals	Mouse	miR-877, 1224, 708, 1981[16–18]	miR-1982[17]
	Rat	miR-877[16]	
	Cow	miR-877[46]	[2, unnamed][46]
	Macaque	miR-877, 1224, 1226, 1227, 1230, 1232, 1235, 1239, 1240, 1241[16]	
	Chimpanzee	miR-877, 1224, 1225, 1226[16]	
	Human	miR-877, 1224, 1225*, 1226, 1227*, 1228, 1229, 1231, 1233, 1234, 1236, 1237, 1238[16,18,47,48]	
Avians	Chicken	[12, unnamed][49]	[6, unnamed][49]

Key references are included. An asterisk indicates those for which evidence indicates they may be alternatively processed via the simtron pathway.

3′ splice site of miR-1003 was shown to largely inhibit the production of pre-miRNA and mature species.[14] Then, allowing recognition of these mutant splice sites through coexpression of a modified version of U1-snRNA, the production of miR-1003 was restored. It was also investigated whether intron debranching is necessary in the mirtron biogenesis pathway. The lariat debranching enzyme performs this function by catalyzing the hydrolysis of the 2′-5′ phosphodiester bond between the branch point and the 3′ end of excised introns. It was found that depletion of this enzyme inhibited the processing of miR-1003, 1006, and 1010, but did not affect canonical miRNAs.[14,15] A similar effect was shown in embryos for miR-1010.[15] Additionally, it was shown that mirtrons can be processed when inserted as introns of a gene other than their endogenous host, proving that there are no specific exonic sequence elements (beyond splice sites) are essential for their processing.[15]

As predicted, *dicer-1* depletion severely reduced the production of mature mirtron miRNA, whereas *drosha* depletion did not.[14] However, a contribution from Drosha in mirtron processing could not be excluded, since the loss of Drosha caused a reduction in mirtron pre-miRNA, although this was only modest compared with the complete loss of *let-7* pre-miRNA. Although this indicates that Drosha is not an essential requirement for mirtron processing, it could be involved in mirtron pre-miRNA stability. The effect could alternatively be explained by an increase in the availability of Dicer to mirtron pre-miRNAs, given the reduced competition with canonical pre-miRNAs in the absence of Drosha. Similarly, a role for Drosha could not be ruled out by Okamura et al., but this factor was again clearly less crucial for mirtrons than for canonical miRNAs.[15] However, in a recent study, mirtron miR-1003 biogenesis was unaffected in *Drosophila drosha* knockout animals.[50] On the other hand, Pasha, the cofactor of Drosha in *Drosophila*, has been consistently proven to play no part in the mirtron pathway. In *pasha* knockout models both mature[50,51] and precursor[51] species were reduced for canonical miRNAs, yet mirtrons were produced.

Involvement of other factors of the canonical miRNA pathway was investigated to further prove that mirtron biogenesis merges with this pathway after splicing and debranching. Like canonical miRNAs, mirtrons were found to be sensitive to knockdown of the nuclear export factor Exportin-5.[15] In addition, direct interaction with dAGO1 was shown by co-immunoprecipitation for two mirtrons, miR-1003 and miR-1010,[15] and depletion of dAGO1 interfered with miR-1010 activity. As expected, interaction with dAGO2, a factor usually involved in non-miRNA pathways in *Drosophila*, was not found.[15]

In order to probe the putative functions of these atypical miRNAs, target knockdown ability was initially proven for miR-1003 and miR-1006 using a luciferase reporter system in *Drosophila* S2 cells.[14] By cloning mirtrons and their endogenous flanking exons into an expression plasmid, strong knockdown of complementary targets was achieved that was comparable to the silencing directed by a *let-7* construct against its analogous target, and strongly suggested that mirtrons are functional as miRNAs. In a similar way, miR-1004 and miR-1010 were shown to specifically knock down fully matched luciferase targets, while seed-region-directed repression was also observed miR-1003 and miR-1004.[15] The knockdown of matched targets was also

investigated for exogenously expressed mirtrons and miRNAs in *pasha* knockout animals alongside *dicer-1* knockout and control animals, which confirmed dependence on Dicer-1 and independence from Pasha for mirtron function.[51]

Finally, having confirmed functionality of processed small RNA species, characteristics of the mature miRNAs have been determined. Although canonical miRNAs can give rise to either 5′- or 3′-derived mature species, the 3′ strand produced the dominant species for all *Drosophila* mirtrons.[14] This was attributed to the fact that mature invertebrate miRNAs rarely have a 5′-G residue,[52] as would be defined by the canonical 5′ splice site. 3′-Strand-derived mirtrons, having no strong constraints at their 5′ end, may begin with the more typical U residue. This flexibility may lead to the tendency for 3′ strand mirtrons to form through evolution in invertebrates by increasing their targeting potential.[14]

A related class of mirtron-like miRNAs have also been confirmed in invertebrates. Specifically, *Drosophila* miR-1017 was classed as a 'tailed mirtron', since only one end of the hairpin (the 5′ end) was found to be derived from the end of an intron (Figures 1(c), 2(b),(c), and 3(c)). Curiously, its 3′ end lies within the intron, meaning that, after splicing, the hairpin would have a single-stranded tail of around 100 nucleotides in length.[19] It was however able to repress reporter genes containing putative target sites to the predicted miRNA. Probing of the pathways involved demonstrated that it was dependent on splicing and the lariat debranching enzyme, but, notably, independent of Drosha/Pasha. Processing of its 3′ tail was instead found to be performed by the 3′-5′ exonuclease activity of the RNA exosome.[19]

In summary, the identification of mirtrons and tailed mirtrons in invertebrates (Table 1) defines a new subclass of miRNAs in which at least one of the hairpin ends is defined by splicing. They are demonstratably independent of normal microprocessor activity but merge with the canonical miRNA pathway at the nuclear export step and require Dicer processing. They are capable of seed-matched target knockdown. However, while no mirtrons could initially be found in the mammalian genome,[14] this has since been shown to be not the case.

Identification and Biogenesis of Mammalian Mirtrons

The first indication of the existence of mammalian mirtrons came from a systematic bioinformatics search. Specifically, Berezikov et al. investigated the existence of mammalian mirtrons with a computational search for short introns having a straight hairpin structure and a saddle-shaped conservation pattern.[16] This conservation pattern means the hairpin-forming ends of the intron are more conserved than the inner (loop) section, as in invertebrate mirtrons and canonical miRNAs.[53] This search revealed 13 well-conserved candidate mirtrons which had hairpin termini corresponding to the predicted splice sites of the short introns. For three of these mirtrons, miRNA-miRNA* reads were found in mouse, rat, chimpanzee, and/or human small RNA libraries. Similar to invertebrate mirtrons, the most highly expressed mammalian mirtrons (miR-877 and miR-1224) were also the most conserved, suggestive of functional relevance. Next, the mammalian brain was thought to be a likely organ to reveal mirtrons given that neurons employ much translational regulation and consistent with this, a diverse set of miRNAs (including mirtrons) was identified in Drosophila heads.[54] RNA libraries of human and macaque brain were therefore searched for reads with similar characteristics. From this, 16 primate-specific mirtrons were confidently classified and a further 46 small RNAs were also considered as candidates (Table 1). Intriguingly, these included a number of tailed mirtrons analogous to *Drosophila* miR-1017. However, both 5′- and 3′-tailed mirtrons were present in this set (Figure 2(b) and (c)).

Since this bioinformatic study, putative mirtrons have also been identified in a number of other mammals, as well as birds and plants, as shown in Table 1. Glazov et al. identified two tailed mirtrons expressed in a bovine cell line, and also found a homolog of miR-877 expressed.[46] Furthermore, Babiarz et al. explored mouse embryonic stem (ES) cells for miRNAs which were enriched in *dgcr8* knockout cells compared with *dicer1* knockout or wild type.[17] Five known miRNAs were seen to be processed without DGCR8, but not without Dicer. One of these (miR-708) appears to be a mirtron, mapping to both ends of its host intron. Using an alternate search approach and instead investigating short introns as a start point, two miRNAs (miR-877 and miR-1981) were identified as DGCR8-independent mirtrons in these cell lines. A tailed mirtron (miR-1982) was also found,[17] thus providing supportive evidence for the processing of this class of miRNAs in mammals. Furthermore, it was shown that the removal of the 11-nt overhang on the 5′ arm was not carried out by Drosha, although the precise exonuclease remains unidentified.

Interestingly, distinct differences between mammalian and invertebrate mirtrons have been found based on the characteristics of the identified sequences.[16] Unlike invertebrate mirtrons, some mammalian mirtrons predominantly produce a mature species from the 5′ end, in cases where the corresponding 3′ species is particularly pyrimidine-rich. However, in cases where mammalian mirtrons were derived from the 3′ end, they were equally likely to begin with C or U, which was not consistent with the complete 5′-U dominance seen in invertebrates. Moreover, unlike the 2-nt 3′-overhang found in canonical miRNAs and invertebrate mirtrons, numerous unusual hairpin end structures are apparent in mammalian mirtrons, the most common being a single-nucleotide overhang on both strands. This may have interesting implications on the recognition mechanisms of Dicer and nuclear export factors.

Although the functionality of mammalian mirtrons was not initially proven, there were numerous positive indicators.[16] The processing of the putative mirtron miRNA:miRNA* duplexes by Dicer was implied by their length being consistent with canonical miRNAs. Furthermore, these duplexes were thought unlikely to be merely degradation products for two reasons. Firstly, searches for duplexes stipulated that RNAs contain 5′ phosphates, a characteristic of processed miRNA species.[55] Secondly, there was a lack of correlation between the number of mirtron reads versus their host mRNA, suggesting that mirtrons may be actively regulated and/or stabilized follow splicing. Finally, the majority of mammalian intron-boundary RNAs were found to derive from short introns, most of them hairpin-forming, which implies that mirtrons and similar introns may have beneficial functional roles which lead them to be actively maintained through mammalian evolution.

In order to probe mammalian mirtron function and biogenesis, we made expression constructs by cloning miR-877, 1224, or 1226 as an intron within the open reading frame (ORF) of enhanced green fluorescent protein (eGFP).[18] The presence of GFP fluorescence therefore shows that mirtrons are accurately spliced from the transcript. As expected, splicing was abolished in identical constructs with single base mutations in either the 5′ splice site or (for miR-877) the branch point. Although a branch point could not be identified for miR-1224 and miR-1226, it may be that alternative branch points are used when one is mutated.[56]

All three mirtrons investigated were found to give knockdown of matched targets cloned into the 3′-UTR of *Renilla* luciferase. The targets were matched to the known dominant strand for each mirtron: the 5′ arm for miR-1224 and miR-877, and the 3′ arm for miR-1226.[16] It was confirmed that knockdown occurred in a splicing- and sequence-dependent manner, by comparison with splicing-deficient or scrambled versions of each mirtron. To further confirm an RNAi mechanism, the precursor species produced in transfected cells were detected by Northern blot and the mature species by deep sequencing. For miR-877, 3′-RACE (Rapid Amplification of cDNA Ends) showed Dicer cleavage sites that corresponded to small RNA reads in deep sequencing libraries. Mirtron expression from the eGFP constructs was unaffected by Drosha knockdown and expression of a dominant negative form of Drosha,[57] unlike canonical miRNAs. Reduced miR-877 target silencing in the presence of viral competitor VA1 suggests dependence on both Exportin-5 and Dicer.[58] Furthermore, this was rescued incompletely by the addition of exogenous Exportin-5, implying that Exportin-5 transports this mirtron hairpin, but Dicer processing is also affected by VA1. It has been shown that Exportin-5 can bind hairpins which bear blunt ends, short (2-nt) overhangs on both strands, or a long 3′ overhang (up to 5-nt).[59] It cannot bind hairpins with long (5-nt) overhangs on both strands. Human miR-877 has a 3-nt 5′ overhang and a 4-nt 3′ overhang, which does not strictly fit into the above group of substrates recognized by Exportin-5. However, given the above result it appears that miR-877 is indeed transported by Exportin-5. Conversely, miR-1224 has single-nucleotide overhangs on both strands, which does fit into the above set, so it would be expected to be a substrate of Exportin-5. However, miR-1224 could not be inhibited by competition with VA1, which suggests that this mirtron could be the subject to an alternative nuclear export route and/or Dicer independence. Although this remains to be fully investigated, Exportins are known to display flexibility through evolution,[60] so it is potentially feasible for a different factor to recognize some mirtron hairpins for nuclear export.

Finally, repression of seed-matched luciferase constructs was shown for multiple putative miR-877 neuronal targets.[18] This was extended to show that levels of the endogenous FXR2 transcript, which contains a seed-matched target region, were reduced in a human neuronal cell line upon overexpression of miR-877. Although it was shown that endogenous miR-877 is present in this cell line, treatment with an antagomir or sponge for this mirtron did not lead to detectable derepression of FXR2, possibly because of the low expression of miR-877 as confirmed by quantitative real time polymerase chain reaction (qPCR).

Excitingly, the first suggestive evidence of a major role for a mirtron in mammalian biology

was recently found by Jones et al. investigating inflammatory lymphangiognesis. Mirtron miR-1236 was found to be upregulated in response to inflammatory cytokines, and it was confirmed through miR-1236 repression, overexpression and target binding that it is a functional regulator of vascular endothelial growth factor receptor 3 (VEGFR-3),[47] thus making this the first bona fide target of a mammalian mirtron. Furthermore, injection of miR-1236 caused inhibition of the lymphangiogenesis and angiogenesis (downstream effects of VEGFR-3) *in vivo*. Given this role, it will be interesting to discover whether there are any cases where this mirtron shows dysregulation directly leading to disease.

In summary, there is clear evidence that the mirtron pathway is functional in mammals. Mature species can be detected, and they may be derived from either arm, and corresponding target knockdown ability can be detected. Their processing is independent of Drosha, but dependent on splicing and cleavage by Dicer. Although mirtrons are largely dependent on Exportin-5 for nuclear export, it is possible that other factors may be involved in some cases. Finally, evidence is now emerging to show that mirtrons play physiological roles in humans, and it will be important to identify further targets of mirtrons in future.

The Simtron Biogenesis Pathway

It was recently shown that two previously predicted mirtrons (miR-1225 and miR-1228) may be alternatively processed via a novel pathway which does not require splicing[26] (Figure 2(i)). These splicing-independent mirtron-like miRNAs, referred to as simtrons, are located within short introns in which the terminal nucleotides of the pre-miRNA hairpin correspond to both the 5′ and 3′ splice sites. However, mature miRNAs are still detected after mutations have been made to the splice-site recognition sequences, preventing splicing of the intron. This curious finding is made even more interesting by the fact that the biogenesis of simtrons appears to be insensitive to manipulations to Dicer, AGO2, and DGCR8, while the production of mature miRNAs is reduced in the presence of a dominant negative Drosha variant. The involvement of Drosha was corroborated through the demonstration that both wild-type and splicing-deficient pre-miRNA could be immunoprecipitated in complex with Drosha, unlike miR-877. However, complete DGCR8 independence cannot be concluded at this stage, especially considering that Drosha characteristically requires DGCR8 presence for targeted cleavage of pri-miRNAs.[9,61] Indeed, wild-type miR-1225 appears to require the presence of both Drosha and DGCR8 in *in vitro* processing assays. Other aspects of the pathway also require clarification. For example, while mature miRNAs from the splicing-deficient variants are reduced by dominant negative Drosha expression, the wild-type variants remain unaffected (miR-1225) or even increase expression (miR-1228) in response to the same treatment. Together these findings could suggest that although the wild-type miRNAs can be recognized by Drosha and processed via the simtron pathway, splicing equally has the capability of processing these miRNAs, and in an endogenous setting may do so before Drosha cleavage occurs. The current evidence indicates that the simtron biogenesis pathway only becomes dominant in splicing-incompetent variants where no competitor processing pathway is present and where mutations may enhance recognition by Drosha. It is unclear what rules apply for these short introns where normal Drosha/DGCR8 recognition signals are not present but splicing signals are competent.[61] Considering that some mirtrons are clearly not subject to Drosha processing even when splicing is inhibited,[18] it appears that a novel Drosha recognition motif exists in a subset of mirtrons. It is also possible that there may be other naturally splicing-incompetent miRNAs that are predominantly processed via this pathway. Some further investigation is therefore required to accurately define the simtron pathway and determine its significance.

REDUNDANCY IN MICRO-RNA BIOGENESIS

The evolutionary basis for diversity in miRNA biogenesis, including the splicing dependence of the mirtron pathway, is not yet clear. One possibility is that the use of the splicing reaction and avoidance of microprocesser activity is in some way beneficial to this subset of mirtron miRNAs such that strong selective pressure maintains their splicing-dependent biogenesis.[17,62] Alternatively, rather than distinct advantages of splicing, it may be that the observed redundancy in splice-site recognition sequences is more amenable for emergence of pre-miRNAs from introns in comparison to the pri-miRNAs,[63] which require a tight structural constraint in order to be recognized by the microprocessor.[9]

Supporting the first hypothesis, the conservation of mirtrons and related pathways across species immediately suggests physiological significance and evolutionary selection pressure. It is known that the miRNA pathway is saturable,[64] with Drosha

processing appearing to be a rate-limiting step.[65] In this regard, the mirtron pathway could have emerged as a means of generating certain important miRNAs using the higher-capacity and non-saturable splicing system. Indeed, recent small RNA profiling from the brain of conditional Dicer and DGCR8 negative mice lends support to the idea that certain miRNAs are being protected.[62] Although the depletion of either protein leads to a lethal phenotype, Dicer-depleted mice display earlier lethality and more severe structural abnormalities relative to DGCR8 loss. Given that ~98% of miRNAs are absent when DGCR8 is lost, this data suggests that miRNAs which avoid the microprocessor, including the mirtrons identified in this study, may have an important role in neural development.

Alternative miRNA pathways may be significant in varying environments within the body. Components of the miRNA pathway are known to show differential expression patterns across several tissues,[66,67] and Drosha orthologs have demonstrated differential expression in lower organisms.[68] Furthermore, under certain conditions including the anaerobic environments within tumors[69,70] and in the presence of certain hormones including estrogen,[71] components of the miRNA pathway can be down-regulated or inhibited which can prevent processing of canonical miRNAs. It will be interesting to see if mirtrons are both present and active in such circumstances, particularly where Drosha and/or DGCR8 are at low levels or absent, and also to see if any mirtrons identified in such settings have fundamental roles that are being protected by their Drosha-independent biogenesis in a physiological setting.

The tightly regulated splicing reaction may provide a further benefit to enhance the evolutionary selection of functionally important mirtrons. Splicing is directed by key regulatory sequences,[72–76] which would in theory limit heterogeneity of the mature miRNA species. Drosha processing is an imprecise step in miRNA maturation and it has been reported that multiple mature miRNAs produced from the same pri-miRNA, which may lead to targeting of alternative gene pools.[77] In the case of miRNAs generated from the 5′ arm of mirtrons, the mature species will begin with the nucleotide defined strictly by the 5′ splice site. Indeed, this is reflected in reads of mature mirtrons within small RNA libraries.[16] The uniformity of the seed region will subsequently implement consistency in the sequences targeted. However, miRNAs derived from the 3′ arm of mirtrons would have their defining 5′ nucleotide determined by Dicer. Crystal structures of Dicer suggest that this enzyme acts as a ruler to measure and cut a defined distance from the base of the stem.[78] At least in the case of miR-1226 this appears to be accurate and reproducible with virtually all species detected in small RNA libraries starting at the same nucleotide.[16] If Dicer processing is indeed more accurate than Drosha processing, then the combination of splicing and Dicer processing would suggest a more uniform population of miRNAs from mirtrons compared with those where Drosha is involved.

There is also compelling support for the alternative hypothesis in which mirtrons have emerged as a consequence of readily mutated short introns evolving into pre-miRNA hairpins, thus bypassing the need for structural recognition by the microprocessor. Most revealing is the fact that all reported mirtrons to date appear restricted to either distinct invertebrate or mammalian orders, in contrast to several canonical miRNAs that show more extensive species conservation which sometimes spans millions of years of evolution.[16,79] This very strongly suggests that the mirtron pathway has emerged independently across multiple arms of evolution and that mirtrons represent a fast way for miRNAs to emerge in the RNAi arsenal. Furthermore, a rapid and independent evolution would offer a ready explanation for the emergence of subtle differences in mirtron characteristics between invertebrates and mammals that have been discussed earlier.[16]

It is likely that neither hypothesis is mutually exclusive. Instead, the suitability of short introns to be readily evolved into pre-miRNAs without the structural constraints of microprocessor recognition has enabled a subset of mirtrons to have emerged across multiple species largely independently and relatively quickly. Subsequently, the attractive advantages of a splicing-dependent biogenesis are likely to be selected for in a subset of important mirtrons, such as those potentially involved in neural development within the mammalian brain.[62]

POTENTIAL APPLICATIONS FOR MIRTRONS IN GENE THERAPY

RNAi strategies, including miRNAs, have previously been designed to direct knockdown of a chosen gene in dominant gain-of-function conditions, being specific enough to target even a single base mutation.[31] Indeed, a number of RNAi clinical trials are underway, including treatments for age-related macular degeneration, viral infection, and some cancers.[80] The mirtron pathway may be similarly exploited in future. Indeed, our work shows that target knockdown achieved by exogenously delivered versions predicted that mirtrons can be comparable to canonical

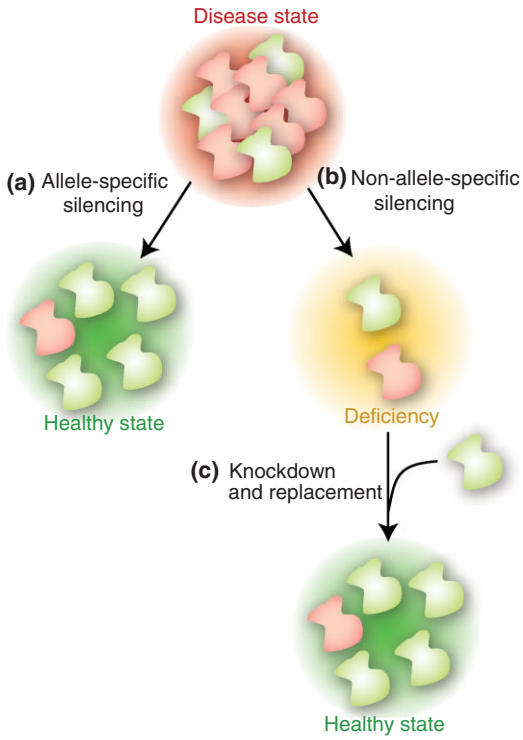

FIGURE 4 | Gene therapy strategies for dominant toxic gain-of-function conditions. In this representation of a dominant condition, in the disease state (top) the mutant (red) protein forms aggregates which also incorporate the wild-type (green) protein. (a) Allele-specific silencing, where possible, reduces levels of mutant protein only, to prevent aggregation while leaving expression of the wild-type allele unaffected. (b) Non-allele-specific silencing may effectively reduce aggregation but lead to deficiency, which may cause known or unknown deleterious effects. (c) Knockdown and replacement add functional protein alongside non-allele-specific knockdown to avoid deficiency.

miRNAs or U6-transcribed hairpins even with lower levels of precursor expression.[18] Furthermore, exploiting this pathway would take advantage of the inherent advantages that this pathway imposes. These include accuracy of production of mature species by splicing, avoidance of the potential bottleneck step of Drosha processing, and convenient combination of RNAi and gene replacement therapy.

A major application where mirtrons may be beneficial is the knockdown and replacement of a gene in which a mutation causes a dominant condition, particularly in cases where an allele-specific knockdown approach is problematic (Figure 4(a)). These include expanded-repeat mutations, for which mature RNAi species are usually too short to distinguish between the expansion and the healthy allele, and conditions with a large degree of genetic heterogeneity, where developing many different mutation-directed therapies is prohibited by cost. In such cases, RNAi could be targeted away from the mutations, hence silencing both alleles (Figure 4(b)). Concurrent delivery of a functional, RNAi-resistant copy of the gene would maintain the protein's physiological role (Figure 4(c)). This knockdown and replacement (K&R) approach could provide a single treatment for all patients affected by the same gene, regardless of their specific mutation. Various approaches to K&R have been investigated for a number of genes, including SCA6,[81] β-globin in sickle cell anemia,[82] rhodopsin in retinitis pigmentosa[83–85] and SOD1 in amyotrophic lateral sclerosis (ALS),[86] summarized in Figure 5.

Using intronic miRNAs in K&R has several advantages. Primarily, intronic miRNA, including mirtrons, may be nestled within a chosen gene, hence providing a simple way to deliver to patients a combination of RNAi and a beneficial gene. Consequently, the gene and miRNA are under the control of the same pol-II promoter, which can be tissue-specific and endogenously controlled. In addition, this would ensure that both aspects of the K&R therapy are always expressed together. Mirtrons would also allow construction of a K&R module of minimal size, which is important since delivery mechanisms have a limited capacity. Considering their biogenesis, mirtrons bypass Drosha, and perhaps also Exportin-5 in some cases.[15,18] These are putative bottleneck steps, where competition can cause toxicity,[64] and so placing minimal dependence on the saturable RNAi pathway can always be considered advantageous wherever possible. Finally, Drosha processing can be subject to variability.[77] By utilizing the splicing pathway which is accurately directed by specific sequence motifs, mirtrons may be processed into more accurate RNAi species, limiting the potential off-target effects.

However, it must be pointed out that synthetic mirtron design presents a number of challenges.[15] Most importantly, the choice of suitable target sites is limited because it must allow the synthetic mirtron to contain the sequence elements necessary for splicing. Nonetheless, the use of synthetic mirtrons for gene therapy will be an interesting area of investigation in future gene therapy application.

CONCLUSION

The mirtron pathway is one of several atypical miRNA biogenesis pathways that have now been identified. Since their discovery a lot has been learnt about mirtrons and their related variants, simtrons, and tailed mirtrons. This includes the identification of nearly 40 mirtrons, 10 tailed mirtrons, and 2 simtrons across 11 different organisms which, for humans at

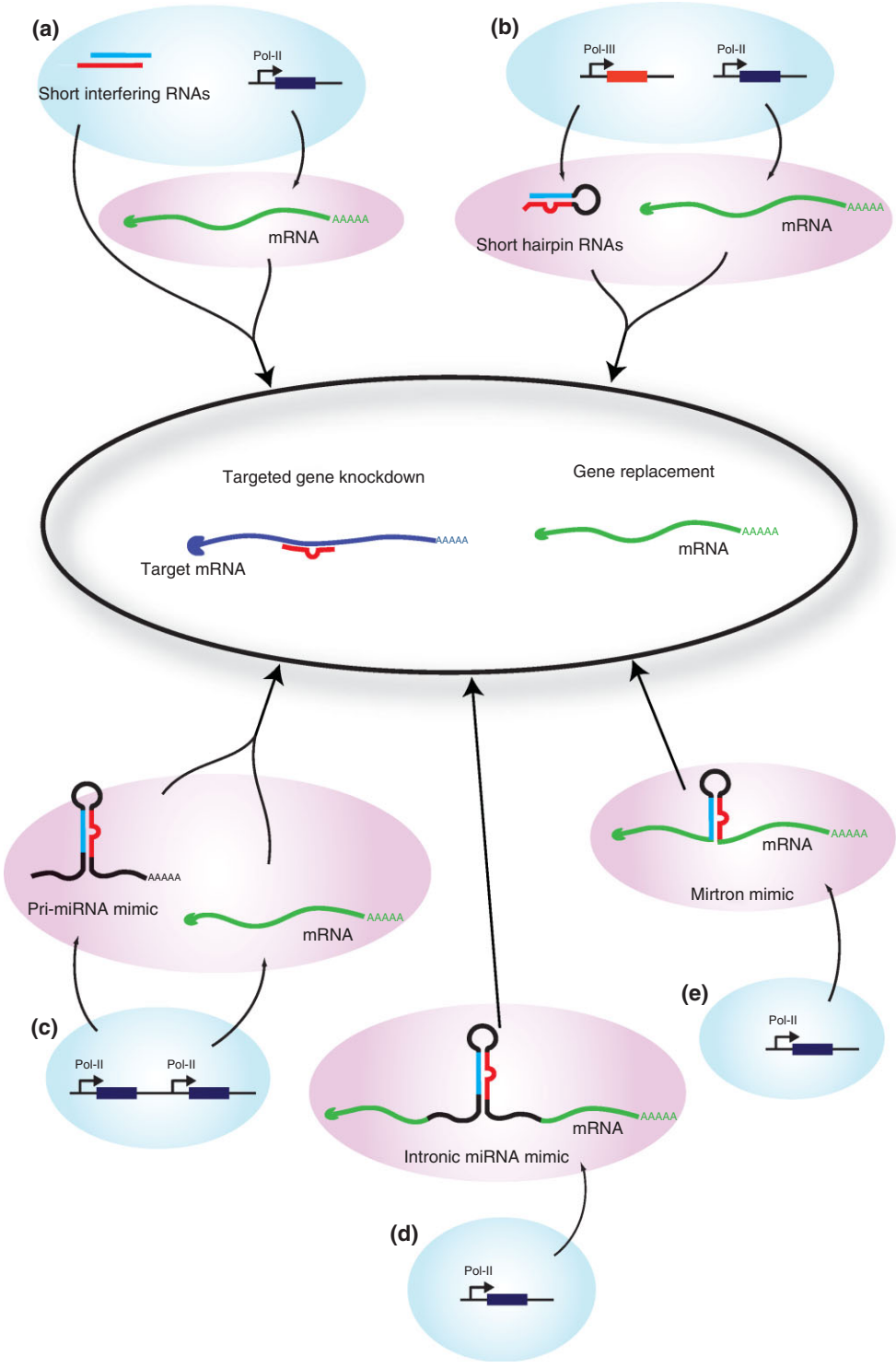

FIGURE 5 | Potential strategies for gene knockdown-replacement (K&R) therapy. (a) Delivery of a targeting siRNA molecule and a transcriptional unit encoding a pol-II/III driven replacement gene.[81,84] (b) Delivery of two transcriptional units: a constitutive pol-III driven shRNA and pol-II/III driven replacement gene.[83,85,86] (c) Delivery of a single construct containing two separate pol-II transcription units: one produces a miRNA mimic and the other produces the replacement gene. (d) A single transcriptional unit driven by a single pol-II promoter where an intronic miRNA mimic is expressed from inside the replacement gene and the transcript is processed by Drosha and splicing.[82] (e) As (d), with a mirtron mimic as a special case of intronic miRNA, which does not require Drosha processing.

least, covers 1–2% of the total miRNA population. Furthermore, significant progress has been made in inferring the putative biogenesis pathways. However, some ambiguity regarding export pathways, nuclease activity, Dicer, and Drosha involvement still require further investigation. In addition, there is a need to experimentally identify the targets of these atypical miRNAs in order to assess their biological significance and relevance to disease, as well as to provide further suggestions as to why these novel biogenesis pathways have emerged. Finally, the pathways detailed here offer new avenues with which to exogenously enter the RNAi pathway, which may confer various advantages. Therefore, while much progress has been made in the study of mirtrons in recent years, there is still much we can learn from this class of atypical miRNA.

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