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REVIEW

Circular RNAs in cell differentiation and development

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ABSTRACT

In recent years, circular RNAs (circRNAs) – a novel class of RNA molecules characterized by their covalently closed circular structure – have emerged as a complex family of eukaryotic transcripts with important biological features. Besides their peculiar structure, which makes them particularly stable molecules, they have attracted much interest because their expression is strongly tissue and cell specific. Moreover, many circRNAs are conserved across eukaryotes, localized in particular subcellular compartments, and can play disparate molecular functions. The discovery of circRNAs has therefore added not only another layer of gene expression regulation but also an additional degree of complexity to our understanding of the structure, function and evolution of eukaryotic genomes. In this Review, we summarize current knowledge of circRNAs and discuss the possible functions of circRNAs in cell differentiation and development.

KEY WORDS: Developmental biology, Differentiation, circRNAs, Noncoding RNAs, Stem cells

Introduction

Circular RNAs (circRNAs) are covalently closed single-stranded RNA molecules that were identified many years ago but have only recently attracted the attention of the general scientific community. They were initially described in the 1970s as the genomic constituents of viroids (Sanger et al., 1976). However, it wasn't until several years later that a connection was made between circRNAs and mRNAs; specifically, it was observed that, upon splicing, not all exons could be found in the order predicted from their positions in genomic DNA (Nigro et al., 1991; Cocquerelle et al., 1993; Starke et al., 2015). As they do not possess polyA tails, circRNAs were missed in transcriptomic profiling studies of polyadenylated mRNAs. Moreover, circRNAs were not identified in total RNA-sequencing experiments because ad hoc computational pipelines identifying back-splicing junctions have only recently been developed (Memczak et al., 2013). Finally, although in a few cases circRNAs can be the predominant isoform produced from a gene (Salzman et al., 2012), they are generally poorly expressed and are present at lower levels than their linear counterpart. It was only with the advent of new techniques for deep RNA-sequencing of total RNA, together with the development of ad hoc computational methods, that circRNAs came to the fore. They were shown to originate from a large number of mRNA coding genes, to be expressed in many cell types and to be conserved across eukaryotes (Salzman et al., 2012; Jeck et al., 2013; Memczak et al., 2013).

Since their discovery, many studies have been devoted to elucidating the expression profiles of circRNAs during development and cell differentiation. These studies have revealed that circRNAs are generally found in the cytoplasm, are expressed in a tissue- and cell-specific manner, and are highly abundant in the nervous system. In addition, and despite the challenges of assessing circRNA function (see Box 1), potential roles for circRNAs, as well their possible alterations in pathological conditions, have emerged. Here, we provide an overview of this research and summarize the key roles of circRNAs in various developmental contexts.

The biogenesis of circRNAs

As mentioned above, circRNAs are usually generated from genes that also produce linear isoforms. They arise via a process termed back splicing, in which an upstream 3′ splice site is joined to a downstream 5′ splice site, resulting in the junction of the 3′ end of an exon with the 5′ end of the same or upstream exon(s) (Fig. 1). The formation of circRNAs can be enhanced by the inhibition or slowing of canonical pre-mRNA splicing events and even by readthrough transcription (Liang et al., 2019; Wang et al., 2019). Even though canonical splicing is usually more efficient than back splicing, specific inhibition of linear splicing, adjuvated by the fact that circRNAs have a longer half-life than their linear counterparts, may allow circRNAs to become the predominant products of their host genes. The fact that canonical splicing and back splicing can be differently affected could be due to differences in exon definition mechanisms and to the requirement of a different set of splicing regulators (Liang et al., 2017; Wang et al., 2019).

One aspect of circRNAs that has been widely investigated is the identification of *cis*- and *trans*-acting factors that control biogenesis. Current research indicates that, similar to alternative splicing, the number of factors that can influence circRNA biogenesis is diverse and complex. Inverted repeats, such as Alu repeats, in the introns flanking the exons to be circularized were the first elements demonstrated to mediate the back-splicing reaction by bringing the splice sites into close proximity (Jeck et al., 2013; Liang and Wilusz, 2014). In some cases, they were shown to be sufficient to trigger back splicing (Zhang et al., 2014). Interestingly, double-stranded RNA sequences produced by Alu elements are particularly susceptible to adenosine-to-inosine (A-I) editing performed by ADAR (adenosine deaminases acting on RNA) enzymes (Kim et al., 2004; Levanon et al., 2004), and A-I editing was found to be enriched in introns surrounding circRNA exons (Ivanov et al., 2015). Because inosine forms Watson–Crick pairing with cytosine instead of thymine, these modifications result in a significant reduction in RNA pairing and a consequent decrease in the production of a subset of circRNAs (Rybak-Wolf et al., 2015). More recently, it was shown that the interferon-inducible isoform of ADAR (p150) is able to interact with DExH-Box Helicase 9 (DHX9), an RNA helicase that binds specifically to Alu elements. Loss of DHX9 leads to an increase in the level and in the number of

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Box 1.

Although a large number of circRNAs has currently been identified, the function and molecular mechanisms of action have been defined for only a few of them. This is in part due to difficulties in studying these molecules, which are sometimes poorly expressed and hardly distinguishable from their linear counterparts. Indeed, the exon(s) included in the circular and linear isoforms share the same sequence. The only feature distinguishing the circularized exon(s) is the back-splicing junction, a peculiarity to be considered when handling repetitive sequences. This feature makes knockdown-based functional studies challenging. Although shRNAs or siRNAs can be designed on the back-splicing junction, the risk of affecting the linear counterpart is strong. Furthermore, circRNAs often derive from coding regions of mRNAs, making their knockout much more complicated than their knockdown. As the circRNA sequence cannot be deleted from the genome without affecting the host gene, removing one of the flanking introns can be a solution. However, large introns can be hard to remove (e.g. by CRISPR-Cas9) because they often host regulatory sequences important for the linear mRNA expression and alternative splicing. Alternatively, the deletion can be restricted to those intronic portions that regulate back splicing but, to date, these cannot be easily predicted.

The sequence identity with the linear counterpart makes also complicates the study of circRNA interactors (mRNAs, miRNAs, proteins). Biotinylated probes spanning the back-splicing junction can be used in RNA pull-downs, but this could be inefficient, and the use of multiple probes poses the problem of contamination of interactors of the linear counterpart.

circRNAs, likely owing to its ability to unwind double-stranded intronic structures or to recruit ADAR. Indeed, this effect is increased if ADAR is also depleted (Aktaş et al., 2017). Although only a small fraction of circRNAs are affected by ADAR depletion (Ivanov et al., 2015), these data point to RNA editing as an important process that regulates the choice between canonical splicing and back splicing.

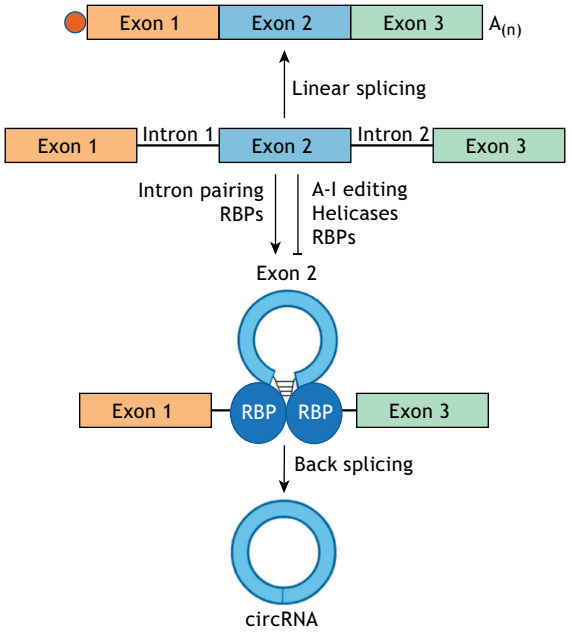

Fig. 1. Linear splicing and back splicing. Schematic of linear splicing (top) and back splicing (bottom). Intron pairings and some RBPs are among the *cis*- and *trans*-acting factors and mechanisms that can enhance back splicing. By contrast, A-I editing, some helicases and other RBPs can inhibit back splicing and hence inhibit circRNA production.

RNA-binding proteins (RBPs) can also regulate exon circularization (Fig. 1). For instance, NF90/NF110 were shown to bind intronic repeats and regulate circRNA production (Li et al., 2017). Unlike DHX9, NF90/NF110 promote circularization by associating with intronic RNA pairs and juxtaposing the circularizing exon(s) (Fig. 1). Another RBP, QKI, was shown to be responsible for the biogenesis of one the most abundant circRNAs in human mammary epithelial cells and to promote circularization by directly binding to flanking introns (Conn et al., 2015). In *Drosophila*, the muscleblind (MBL) protein was shown to autoregulate its own production in a negative-feedback loop involving a circRNA (circMBL) originating from the same gene. In fact, although MBL promotes the formation of the circular form at the expense of the linear one, it also remains bound to it, thus lowering the levels of free MBL protein and bringing linear splicing levels back to normal (Ashwal-Fluss et al., 2014). The ALS-associated protein Fused in Sarcoma (FUS) is another RBP that can regulate the production of circRNAs. It has been shown in mouse embryonic stem cells to bind to the neighboring intron region of the exons to be circularized and to regulate the expression of 132 circRNAs without affecting the levels of their linear counterparts (Errichelli et al., 2017). Heterogeneous nuclear ribonucleoproteins (hnRNPs) and serine/arginine-rich (SR) proteins have also been shown to participate in circRNA biogenesis (Ashwal-Fluss et al., 2014; Kramer et al., 2015; Errichelli et al., 2017), as have specific splicing factors such as ESRP1, which controls the circularization of circBIRC6 by binding to specific sites in the introns flanking the circularizable exon (Yu et al., 2017).

Interestingly, the lariat structure – a splicing intermediate with a branched circular conformation – can act similarly to inverted repeats and RBPs by favoring the circularization of sequences contained within it (Holdt et al., 2018). Although the lariat structure itself cannot account for circularization, exon-retaining lariats occasionally have the ability to produce circRNAs, and longer retained exons were shown to circularize more than shorter ones (Barret et al., 2015).

More recently, RNA modifications have also been shown to play a role in the biogenesis of circRNAs. In particular, for a specific subset of circRNAs, N6-methyladenosine (m^6A) deposition at specific sites on primary transcripts was shown to direct the splicing machinery towards the back splicing reaction through a circuitry involving the METTL3 m^6A writer and the YTHDC1 m^6A reader (Di Timoteo et al., 2020).

General mechanisms of action

circRNAs have been found to be broadly expressed, conserved and modulated in response to cellular stimuli, and are sometimes expressed in a tissue-specific fashion (Salzman et al., 2012; Jeck et al., 2013; Memczak et al., 2013). In most cases, circRNAs exhibit cytoplasmic localization, although few details are available about the factors involved and mechanisms through which they are exported in the cytoplasm (Huang et al., 2018; Chen et al., 2019a). Current evidence suggests that circRNAs can actually have biological relevance and specific molecular activities (Fig. 2, Table 1). However, despite the large number of identified species, the characterization of their molecular mechanism of action is in most cases still largely owing due to difficulties in experimentally analyzing circRNA function (see Box 1).

The first example of an active circRNA was CDR1as, which is preferentially expressed in the brain (Hansen et al., 2013a,b; Memczak et al., 2013; Piwecka et al., 2017). CDR1as, which is sometimes referred to as ciRS-7, CDR1-AS or CDR1os, was

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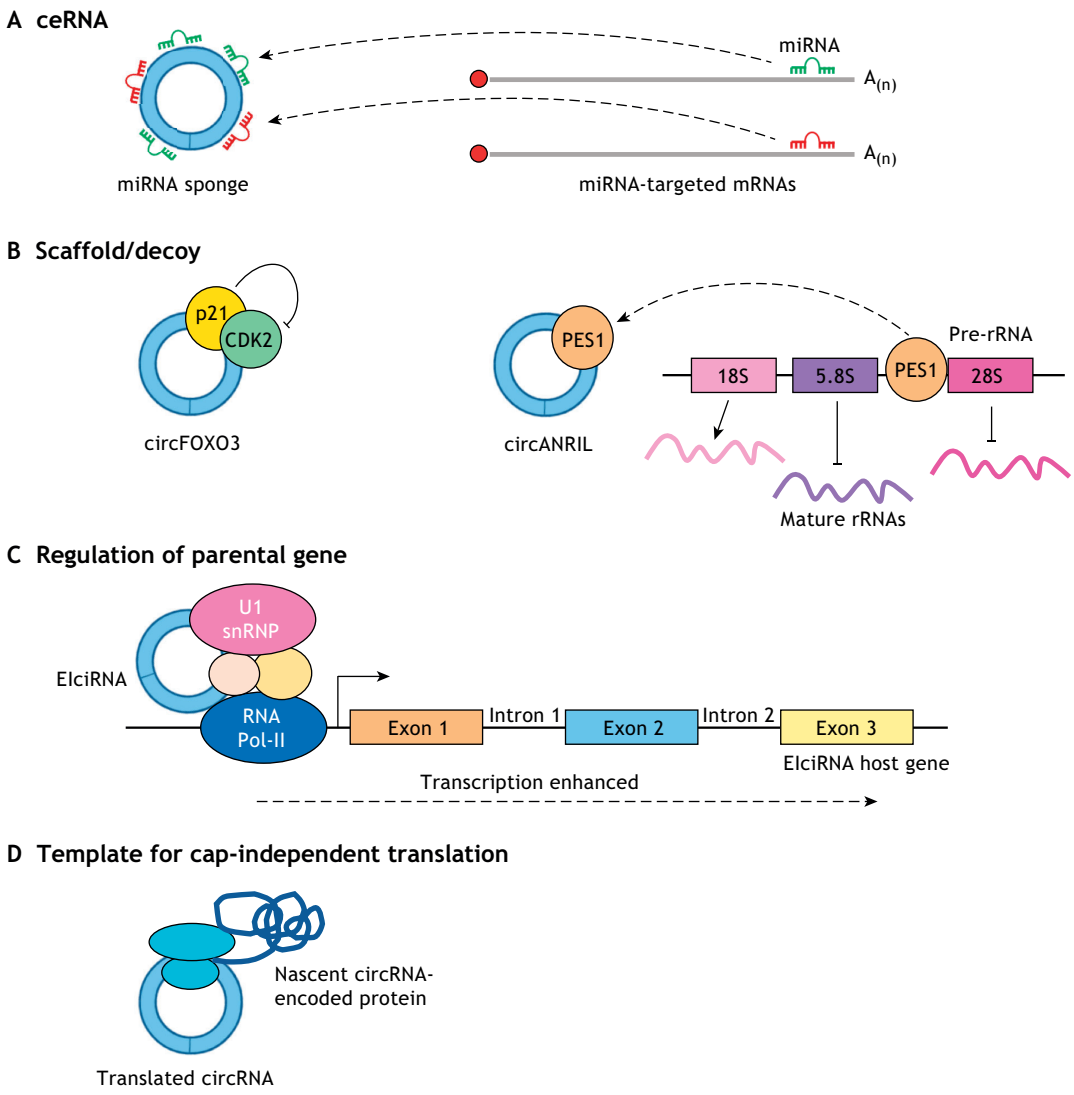

Fig. 2. Molecular functions of circRNAs. (A) circRNAs can act as competing endogenous RNAs (ceRNAs) by sequestering and/or stabilizing miRNAs. (B) circRNAs can act as scaffolds or decoys for RBPs; the examples of circFOXO3 and circANRIL are shown here. (C) circRNAs can also regulate the transcription of their parental gene, as occurs in the case of circPAIP2 and circEIF3J. RNA Pol-II, RNA polymerase II. (D) Some circRNAs can act as a template for cap-independent translation.

initially considered to act as a competing endogenous RNA (ceRNA; Fig. 2A) by sequestering multiple copies of the microRNA miR-7 and interfering with its mRNA-targeting activity (Memczak et al., 2013). However, in a more recent work, the ceRNA activity of CDR1as has been questioned (Piwecka et al., 2017). Following the establishment of a CDR1as knock-out mouse model, it was shown that the loss of CDR1as was related to the dysfunction of excitatory synaptic transmission, and also to a downregulation of miR-7 and an upregulation of its target mRNAs at the molecular level. This finding was in contrast with the previously proposed miRNA-sponge activity of CDR1as and highlighted a more complex role for this circRNA. In particular, the authors proposed that CDR1as may instead stabilize rather than titrate miR-7 and translocate it towards synapses (Piwecka et al., 2017). Although the role of circRNAs as ceRNAs cannot be generalized, there are several examples to date of circRNAs that alter microRNA activity and impact many cellular functions, primarily those related to cell proliferation (Li et al., 2015a; Zheng et al., 2016; Zhong et al., 2017; Panda, 2018; Zhou et al., 2020; Zang et al., 2020). For instance, another circRNA with multiple miRNA binding sites is Sry, a testis-specific circRNA that acts by sponging miR-138 (Capel et al., 1993; Hansen et al., 2013a).

circRNAs can also act as scaffolds or decoys for RBPs (Fig. 2B). One of the most interesting cases is circFOXO3, which forms a ternary complex with the proteins p21 (CDKN1A) and CDK2; the effect of this assembly is that the function of CDK2 is arrested with the consequent block of cell cycle progression (Du et al., 2016). Through a similar mechanism, circANRIL sequesters PES1, suppressing ribosome biogenesis in smooth muscle and in macrophages (Holdt et al., 2016).

Another example of functional circRNAs is represented by the so-called ‘Exon-Intron’ circRNAs (EIciRNAs; Fig. 2C). Examples of these are circPAIP2 and circEIF3J, which control the expression of their mRNA counterparts through the binding of small nuclear ribonucleoproteins (snRNPs) and the modulation of RNA polymerase II activity (Li et al., 2015b). In particular, it was shown that EIciRNAs can base-pair with U1 snRNA, and that contact with RNA polymerase II can be mediated by U1 snRNP.

Some circRNAs have also been shown to act as templates for cap-independent translation (Fig. 2D), sometimes enhanced by the m^{6}A

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Table 1. circRNA functions

circRNA	Cell type	Biological function	Molecular function	References
CDR1as	Neural tissue, PDLSCs, goat satellite muscle stem cells	Neural development, osteoblast differentiation, muscle stem cell differentiation	miR-7 regulation	Memczac et al., 2013; Li et al., 2018b, 2019a
SRY	Testis	Testis development	Protein coding, miR-138 sponge	Hansen et al., 2013a
circBIRC6	hESCs	Pluripotency	miR-34a sponge, miR-145 sponge	Yu et al., 2017
circCORO1C	hESCs	Pluripotency	–	Yu et al., 2017
circFOXP1	MSCs	Bone repair	Suggested miRNA sponge	Cherubini et al., 2019
circHomer1_a	Rat primary hippocampal neurons	Synaptogenesis	–	You et al., 2015
circIGSF11	Human BMSC-derived osteoblasts	Osteoblast proliferation	Suggested miR199b-5p sponge	Zhang et al., 2019
hsa-circRNA_0074834	Human BMSC-derived osteoblasts	Osteoblast differentiation	Suggested miR942-5p sponge	Ouyang et al., 2019
circPOMT1	Human ADSC-derived osteoblasts	Osteoblast proliferation	Suggested hsa-miR-6881-3p sponge	Huang et al., 2019b
circMCM3AP	Human ADSC-derived osteoblasts	Osteoblast proliferation	Suggested hsa-miR-6881-3p sponge	Huang et al., 2019a,b
circRFWD2	Human ADSC-derived osteoblasts	Osteoblast differentiation	Suggested hsa-miR-6817-5p sponge	Huang et al., 2019a
circINO80	Human ADSC-derived osteoblasts	Osteoblast differentiation	Suggested hsa-miR-6817-5p sponge	Huang et al., 2019a
mmu_circRNA_013422	Murine ADSC-derived osteoblasts	Osteoblast differentiation	Suggested miR-338-3p sponge	Long et al., 2018
mmu_circRNA_22566	Murine ADSC-derived osteoblasts	Osteoblast differentiation	Suggested miR-338-3p sponge	Long et al., 2018
circBANP	PDLSCs	Osteoblast differentiation	Suggested miR-34a and miR-146a sponge	Gu et al., 2017
circITCH	PDLSCs	Osteoblast differentiation	Suggested miR-34a and miR-146a sponge	Gu et al., 2017
circQKI	Human primary myoblasts	Myoblast differentiation	–	Legnini et al., 2017
circBNC2	Human primary myoblasts	Myoblast proliferation	–	Legnini et al., 2017
circZNF609	Human primary myoblasts murine myoblasts	Myoblast proliferation	Protein coding, suggested miR-194-5p sponge	Legnini et al., 2017
circFUT10	Bovine myoblasts	Myoblast proliferation	miR-133a sponge	Li et al., 2018a
circLMO7	Bovine myoblasts	Bovine myoblast proliferation	Suggested miR-378a-3p sponge	Wei et al., 2017
circZNF91	Human epidermal cells	Keratinocyte differentiation	miR23b-3p and miR-766-3p sponge	Kristensen et al., 2018
circH19	ADSCs	Adipogenesis inhibition	PTBP1 binding	Zhu et al., 2020

–, unknown function.

RNA modification (Legnini et al., 2017; Pamudurti et al., 2017; Yang et al., 2017; Di Timoteo et al., 2020). These circRNAs harbor an open reading frame (ORF) spanning the back-splicing junction until they reach a downstream termination code. Therefore, a new ORF is produced that differs from that of the corresponding linear counterpart in the codons arising downstream of the back-splicing junction (Fig. 2). However, to date, a coding ability has only been reported for a few circRNA species: circZNF609, which is expressed in human primary myoblasts and controls cell proliferation (Legnini et al., 2017); circMBL, which is produced in the *Drosophila* head (Pamudurti et al., 2017); circSHPRH, the peptide of which has a suppressive role in glioblastoma (Begum et al., 2018); and circβ-catenin, which has a role in controlling the growth of liver cancer cells through the Wnt pathway (Liang et al., 2019).

Finally, circRNAs have recently been proposed to act as key modulators of the innate immune response (Fig. 3). Endogenously produced circRNAs can be recognized as 'self' molecules by cytoplasmic immune receptors (i.e. RIG-I, also known as DDX58) as they are marked by RNA modifications (i.e. m^6A) and are bound to specific cellular proteins (Chen et al., 2017, 2019b; Fig. 3A). These self circRNAs can then regulate antiviral responses by competing with viral mRNAs for binding to NF90/NF110 factors (Li et al., 2017), or by inhibiting the activity of protein kinase R (PKR, also known as EIF2AK2), which plays a role in the early stress response

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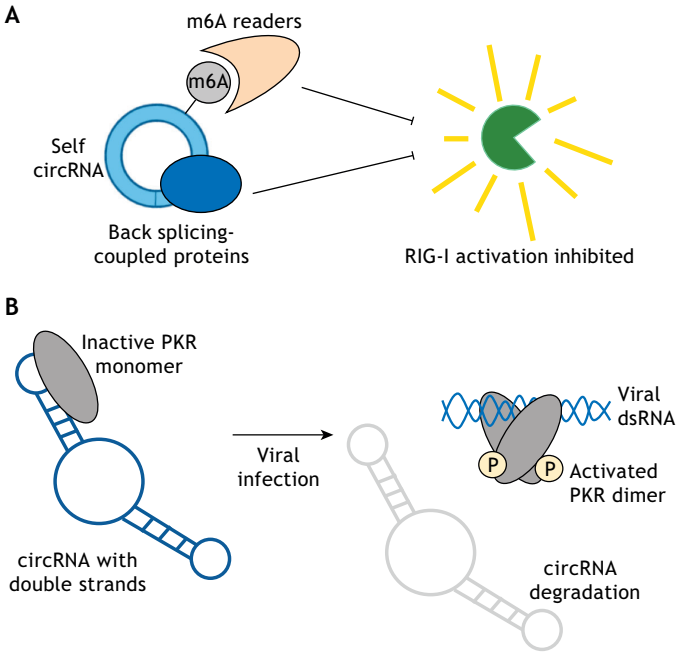

Fig. 3. circRNAs can regulate the innate immune response. (A) Endogenous circRNAs produced by cellular back splicing do not induce RIG-I activation because they can be recognized as 'self' molecules (due to the binding of back splicing-coupled proteins, m6A decoration and m6A-reader recognition). By contrast, circRNAs devoid of this 'self' signature can activate RIG-I and hence induce an innate immune response. (B) Endogenous circRNAs harboring specific secondary structures can bind inactive PKR monomers, discouraging activation of the anti-viral response. Upon viral infection, circRNAs are globally degraded and PKR monomers are released and participate in an anti-viral response.

(Liu et al., 2019b; Fig. 3A). According to these data, naked exogenous circRNAs boost the innate immune response through the activation of immune receptors (i.e. RIG-I) (Chen et al., 2017). However, recent evidence ascribes the immunogenicity of these molecules to their imperfect purification, caused by linear RNA contamination, showing that pure naked exogenous circRNAs are able to evade innate immune receptors (Wesselhoeft et al., 2019).

circRNAs in stemness

Besides being abundant in undifferentiated human embryonic stem cells (hESCs) and being associated with pluripotency (Yu et al., 2017), specific circRNAs have been shown to promote and maintain hESC pluripotency. For example, circBIRC6 and circCORO1C knockdown impairs pluripotency, whereas the ectopic expression of these circRNAs in hESCs promotes pluripotency and enhances the efficiency of induced pluripotent stem cell (iPSC) generation (Yu et al., 2017). In particular, circBIRC6 was shown to bind to the RNA-induced silencing complex (RISC) and to function by inhibiting miR-34a- and miR-145-mediated suppression of the pluripotency factors NANOG, OCT4 (POU5F1) and SOX2, thus favoring their expression and maintaining the pluripotent state. Interestingly, circBIRC6 biogenesis was shown to be controlled by the alternative splicing regulator ESRP1, the expression of which in turn is controlled by NANOG and OCT4. Therefore, this is an interesting regulatory circuitry for the control of stemness, which involves the interplay of pluripotency factors, splicing factors, circRNAs and miRNAs (Yu et al., 2017; Fig. 4).

Mesenchymal stem cells (MSCs) have a circRNA expression profile that is very different to that of differentiated fibroblasts (Cherubini et al., 2019). In particular, circFOXP1 is upregulated in MSCs compared with all *in vitro*-generated mesodermal derivatives and human biopsies of bone, cartilage and adipose tissue. Moreover, its downregulation reduces MSC growth, indicating circFOXP1 as a marker of undifferentiated MSCs. *In vivo*, the knockdown of circFOXP1 in MSCs injected into the rat femur of an atrophic non-union model – an animal model of nonhealed femur factures – determines the lack of bone union development, demonstrating a role for circFOXP1 in bone repair. Based on these findings, circFOXP1 was suggested to regulate these different molecular pathways by acting as a ceRNA for multiple miRNAs (Cherubini et al., 2019).

Furthermore, circRNAs have been demonstrated to be abundant in male germ cells. The ratio of circular versus linear products increases during spermatogenesis for a large number of circRNAs. Interestingly, recent evidence such as the presence of large potential ORFs, m^6A-modified start codons (associated with translation) and the association with polysomes during cell development suggested that a subset of these circRNAs might encode for proteins, and mass spectrometry analysis has indeed proved the coding ability of such molecules (Tang et al., 2020). Taking advantage of a sequence-based bioinformatic approach, a recent study provided a comprehensive dataset of circRNA/miRNA interactions in mouse germline stem cells (Li et al., 2019b). Although functional analyses of these interactions remain to be performed, these data unveil new potential circRNA/miRNA regulatory circuitries and has opened circRNA research up to studies of germ cell development and human reproduction.

circRNAs in neurogenesis

circRNAs have been shown to be abundant in neuronal tissues, to be upregulated during neuronal differentiation and to accumulate with age (Rybak-Wolf et al., 2015; Venø et al., 2015; You et al., 2015; Westholm et al., 2014; Yang et al., 2018). In cell culture models of neural differentiation, for instance, the expression of the majority of circRNAs was shown to be significantly upregulated and to vary during differentiation (Rybak-Wolf et al., 2015).

Very recently, the depletion of circSlc45a4, a highly conserved circRNA, was shown to not only induce spontaneous neuronal differentiation in a human neuroblastoma cell line but also to produce a significant reduction in the basal progenitor pool in the developing mouse cortex and a decrease in cells in the cortical plate together with an increase in Cajal–Retzius cells, which are neurons involved in brain development (Suenkel et al., 2020). These results highlight the role of circSlc45a4 in cortex formation and in the maintenance of the pool of neural progenitors both *in vitro* and *in vivo*.

Hundreds of circRNAs are highly and specifically expressed in the brain tissues of mouse, human, rat and pig. Notably, 80% of all efficiently expressed mouse neuronal circRNAs are also detected in the human brain, with very similar expression patterns seen across neuronal tissues (Rybak-Wolf et al., 2015). Moreover, approximately 20% of the splice sites involved in porcine circRNA production are functionally conserved between mouse and human (Venø et al., 2015). Deep sequencing of porcine brain samples has demonstrated that circRNAs are highly abundant and dynamically expressed in a spatiotemporal manner (Venø et al., 2015). For example, high levels of circRNA expression are observed in the cortex during early to mid-gestation, which is when neurogenesis occurs. Interestingly, the circRNAs expressed derive from genes related to axon guidance, Wnt signaling and the TGFβ signaling pathway, i.e. factors that strongly impact neuronal differentiation and migration (Yi et al., 2010; Salinas, 2012; Rosso and Inestrosa, 2013; Stipursky et al., 2014).

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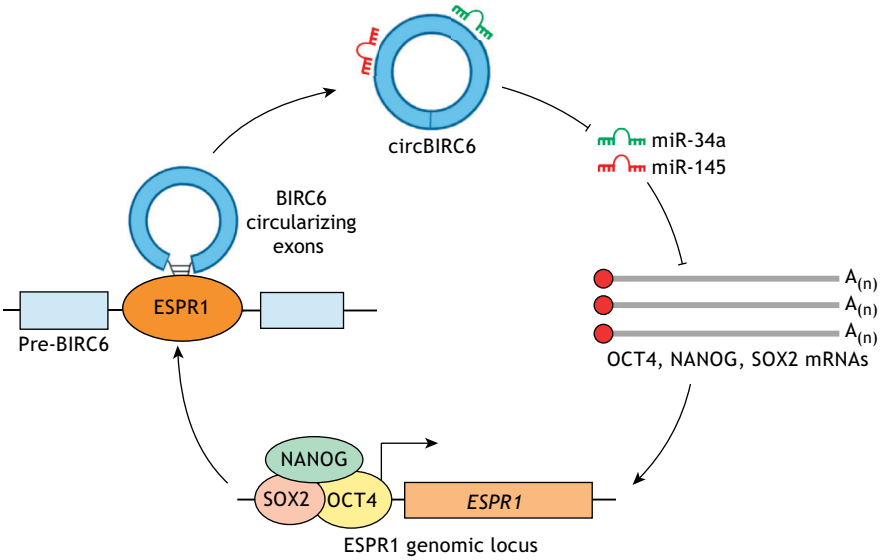

Fig. 4. circBIRC6 sustains pluripotency and its own expression through a positive-feedback loop. circBIRC6 sequesters miR-34a and miR145, releasing their targets (OCT4, NANOG and SOX2 mRNAs) from repression. NANOG, OCT4 and SOX2 proteins sustain the expression of the *ESPR1* gene by binding to its promoter. ESPR1 protein, in turn, sustains circBIRC6 production by positively regulating its back splicing.

Beyond CDR1as, which we introduced above, a number of other circRNAs have been investigated in neuronal tissues. For example, circRIMS2 – a circRNA specifically expressed in the developing embryonic pig cortex – is also highly expressed in the human cortex, whereas it is almost exclusively located in the cerebellum of the murine brain (Rybak-Wolf et al., 2015). This indicates that organism-, tissue- and developmental stage-specific regulatory mechanisms are fundamental for the control of circRNA levels in the brain. A possible involvement of circRNAs in synaptogenesis has also been suggested. Indeed, it has been shown that circRNA expression dramatically changes at the beginning of this process in mice (You et al., 2015), and that circRNAs produced by synapse-related genes (Venø et al., 2015; You et al., 2015) are upregulated during the establishment of mature neural circuits (You et al., 2015). Furthermore, circRNAs are highly enriched in synaptoneurosomes (You et al., 2015) and their compartmentalization differs from that of their linear cognates (Venø et al., 2015). For example, circStau2a is mainly present in synapses, whereas its linear counterpart primarily locates to the cytoplasm (Venø et al., 2015). Interestingly, one particular circRNA (circHomer1_a; encoded by the *Homer1* gene) that is known to play a key role in postsynaptic density regulation was the most significantly upregulated circRNA after induction of synaptic plasticity (Meyer et al., 2014; You et al., 2015). Interestingly, circHomer1 has been associated with psychiatric disorders, such as schizophrenia and bipolar disorder. circHomer1 *in vivo* depletion not only impaired orbifrontal cortex-mediated cognitive flexibility in mouse orbifrontal cortex but also caused the deregulation of genes involved in both synaptic plasticity and psychiatric disorders in the prefrontal cortex (Zimmerman et al., 2020).

Although the precise function of the large set of neuronal circRNAs is, in most cases, still unknown, it is possible to envisage that they may control synaptic function by serving as scaffolds for proteins and mRNAs or by acting locally as miRNA sponges (Hansen et al., 2013a,b; Rybak-Wolf et al., 2015; Zheng et al., 2016). Moreover, they may be involved in controlling intracellular trafficking in axons and dendrites, and the localization of proteins and mRNAs at synaptosomes, or they may be secreted at the synapse level and function in signal transmission. The intrinsic stability of circRNAs makes them ideal molecules for storing information and for making it available in response to different external stimuli.

circRNAs in osteogenesis

Bone development is a complex process that starts during embryonic development and is completed during postnatal life. Moreover, bone fractures can be healed thanks to the reactivation of osteogenic mechanisms. A number of different transcriptional and post-transcriptional mechanisms modulate osteoblast differentiation, as well as bone size and shape (Berendsen and Olsen, 2015). Recently, circRNAs have been added to this list of modulators of bone development. The differential expression and possible molecular activities of circRNAs have been reported during osteoblast differentiation of adult stem cells such as bone marrow stem cells (BMSCs) and adipose-derived stem cells (ADSCs).

Regarding the role of circRNAs in BMSC osteogenesis, there is evidence for a functional circRNA in the context of bone tissue regeneration during fracture healing (Ouyang et al., 2019). In particular, the study focused on a pathological condition called nonunion, in which bone fractures cannot heal spontaneously. This work highlighted profound differences in circRNA expression patterns between controls and nonunion patients. Among the modulated circRNAs, hsa-circRNA_0074834, deriving from the *TTC1* gene, was found to be upregulated during BMSC osteoblast differentiation but downregulated in the pathology. This circRNA was clearly shown to regulate osteogenesis positively by acting as a ceRNA for miR-942-5p, thus providing a good example of a ceRNA that functions during bone regeneration. Among the targets of miRNA-942-5p were ZEB1 and VEGF mRNAs, which are involved in the regulation of both osteogenesis and osteogenesis-angiogenesis coupling, suggesting that this circRNA-miRNA-mRNA circuit can control bone regeneration during fracture healing (Ouyang et al., 2019).

ADSCs are another type of mesenchymal cell that can give rise to osteoblasts. They are particularly useful in bone regenerative medicine because they can be more easily obtained (e.g. from adipose tissue with respect to bone marrow derivation) and have a higher proliferation and differentiation potential than BMSCs (Storti et al., 2019). Differential expression of circRNAs has been observed during human ADSC osteogenesis (Huang et al., 2019b). circPOMT1 and circMCM3AP are among the downregulated species. Their depletion favors ADSC-derived osteoblast differentiation and their expression trend negatively correlates with that of hsa-miR-6881-3p. In addition, both of these circRNAs

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were predicted to inhibit miRNA activity of the transcripts of SMAD6 and chordin, two factors that regulate osteogenesis through the BMP signaling pathway (Huang et al., 2019b). The role of circPOMT1 and circMCM3AP in regulating activity of the same miRNA is interesting, as it points out the importance of cooperative circuits involving either more than one circRNA and the same miRNAs, or one circRNA sponging different miRNAs with the same targets. These cooperative circuits can corroborate the ceRNA activity of a circRNA, especially when it is not highly expressed or when it harbors a few binding sites for a specific miRNA.

Another example of cooperative circuit, in which two circRNAs sponge the same miRNA, is represented by circRFWD2 (RFWD2 is also known as COP1) and circINO80 (Huang et al., 2019a). They are upregulated upon the induction of NELL1, a protein factor that plays an important role in sustaining osteogenesis (Liu et al., 2012). circRFWD2 and circINO80 knockdown impairs NELL1-induced osteogenesis, suggesting that they are downstream modulators of its signaling. These two circRNAs sustain osteoblast differentiation by sponging hsa-miR-6817-5p, a negative regulator of this process (Huang et al., 2019a,b). However, the pathways affected by the sponging of this miRNA have not yet been clarified; therefore, this circRNAs-miRNA interaction needs further investigation.

Besides ADSCs and BMSCs, an emerging model for bone development and regeneration is represented by periodontal ligament stem cells (PDLSCs), which have a crucial role in supporting osteoblast differentiation. PDLSCs are useful for studying the formation of periodontal and alveolar bone tissues, but also some nerves and blood vessels (Gu et al., 2017; Zheng et al., 2017). The differential expression of circRNAs has been analyzed in the context of PDLSC osteogenic differentiation, and some regulatory networks have been proposed (Gu et al., 2017; Zheng et al., 2017; Li et al., 2018b). One of them includes the long non-coding RNAs (lncRNAs) TCONS_00212979 and TCONS_00212984, as well as circBANP and circITCH. All four of these RNAs can bind miR-34a and miR-146a, thus upregulating some of their target mRNAs involved in the MAPK pathway (DUSP1, FAS, RAC1, PDGFRA, TGFBR2 and MYC) (Gu et al., 2017). The well-studied CDR1as/miR-7 axis also plays a role in PDLSCs. In fact, CDR1as can promote osteogenesis by avoiding miR-7-mediated repression of GDF5. The latter, in turn, sustains PDLSC differentiation by promoting SMAD1/5/8 phosphorylation and MAPK pathway activation (Li et al., 2018b).

In addition to these studies focusing on circRNAs with a functional relevance to osteogenesis, there are others confirming the modulation of circRNA expression during osteoblast differentiation. For example, a microarray-based analysis of circRNA expression revealed that thousands of circRNAs are deregulated during osteoblast differentiation, the majority of which come from genes involved in osteogenesis (Zhang et al., 2019). In this work, a regulatory network involving circIGSF11 and miR199b-5p was hypothesized, based only on the observations that circIGSF11 has one binding site for miR199b-5p, that there is an inverse correlation between these two molecules during osteogenesis, and that circRNA knockdown promotes upregulation of miR-199b-5p (Zhang et al., 2019). However, this molecular circuit has not yet been further investigated, and the ceRNA activity of circIGSF11, as well as its effects on osteoblast differentiation, remain to be clarified. Indeed, circIGSF11 has only one binding site for miR-199b-5p, so it could exert limited sponging activity. Therefore, given the lack of strong evidence, the functional relevance of circIGSF11 and its hypothesized miRNA-sponge activity remain questionable.

Just as circRNAs can contribute to osteogenesis of human ADSCs, they also participate in the same process in mouse. From circRNA-miRNA co-expression network analysis, miR-338-3p emerged as an important node, showing an inverse correlation with mmu_circRNA_013422 and mmu_circRNA_22566. As miR-338-3p can potentially bind the two circRNAs and is known to target two key regulators of osteogenesis (Fgfr2 and Runx2), the authors proposed that a mmu_circRNA_013422/22566-miR-338-3p - Fgfr2/Runx2 circuit could act as a fine-tuning network during osteoblast differentiation (Long et al., 2018).

circRNAs in skeletal muscle development

Myogenesis is a highly regulated process, the success of which is ensured by a delicate equilibrium of transcription factors and non-coding RNA activities (Bentzinger et al., 2012; Zhao et al., 2019). In recent years, some circRNAs have been described to function alongside lncRNAs and miRNAs as novel regulators of skeletal muscle growth and differentiation.

Early studies of circRNAs in muscle differentiation identified 2100 and 1600 circRNAs that are expressed and modulated during human and murine myogenesis, respectively, with almost 600 of these being conserved between the two species (Legnini et al., 2017). Based on a large siRNA-based phenotypic screen, some of these circRNAs were shown to have a role in regulating different aspects of muscle development. For example, circQKI (as well as QKI mRNA) depletion was demonstrated to have a negative effect on myoblast differentiation, indicating that both the circRNA and its linear counterpart cooperate in this process. By contrast, although BNC2 mRNA depletion causes an increase in myotube formation, knockdown of its circular counterpart has no effect on differentiation. Interestingly, circBNC2 expression during myoblast differentiation increases at the expense of the corresponding mRNA, suggesting that circBNC2 could contrast the expression of the anti-differentiative BNC2 mRNA (Legnini et al., 2017).

A third interesting circRNA described to function in muscle development is circZNF609 (Legnini et al., 2017). It is expressed in human growing myoblasts, and its levels decrease upon differentiation. Its role in sustaining myoblast growth was confirmed by the evidence that its specific depletion strongly affects human primary myoblast proliferation (Legnini et al., 2017). Moreover, circZNF609 was recently discovered to be upregulated in a skeletal muscle-derived tumor (rhabdomyosarcoma), in which its knockdown induces an evident cell cycle slow-down at the G1-S transition (Rossi et al., 2019). Consistent with its role in supporting cell proliferation, circZNF609 was also found to be upregulated in Duchenne muscular dystrophy, which is characterized by a higher percentage of growing myoblasts at its early stages (Legnini et al., 2017). Another characteristic of circZNF609 is that it can be translated into two proteins. In fact, its sequence hosts two start codons (AUGs), shared with the ORF of ZNF609 mRNA. Upon circularization, the two AUGs are in-frame with a stop codon downstream of the back-splicing junction, giving rise to a circRNA-specific ORF that can actually be translated (Legnini et al., 2017). So far, the role of these circZNF609-encoded proteins in myoblast proliferation and differentiation is unknown, hence further studies are required.

More recently, circZNF609 has also been proposed to suppress myotube differentiation in the C2C12 murine muscle cell line via the sponging of miR-194-5p (Wang et al., 2019). It was observed that, upon circZNF609 knockdown, the expression of some myogenic markers (such as MYF5 and MYOG) increases,

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whereas the levels of BCLAF1, a negative regulator of myogenic differentiation and a target of miR-194-5p, decrease (Wu et al., 2019). However, the presence of only one miRNA-194-5p binding site in circZNF609, the absence of its conservation in human, and the relatively low abundance of circZNF609 suggest that a role for direct ceRNA activity in this context needs to be further and more deeply investigated.

Besides functioning in human and mouse muscles, circRNAs have been found to regulate skeletal muscle development in other species. Because studying muscle biology is also relevant for the food industry and for meat production, some studies have focused on molecular processes regulating livestock myogenesis. These studies have revealed that there are circRNAs playing a role in bovine and goat myogenesis. For example, circFUT10 sustains bovine myogenic differentiation, inducing the exit of myoblasts from the cell cycle and their accumulation in G0/G1 phase, by sponging miR-133a (Li et al., 2018a). By contrast, circLMO7 was suggested to sustain bovine myoblast proliferation. Indeed, its overexpression increases the proportion of cells in S phase, while inhibiting their differentiation (as shown by a decrease in MYOD and MYOG expression) and counteracting apoptosis (Wei et al., 2017). In goat mid-embryonic muscle tissue, expression of the well-known circRNA CDR1as can be transcriptionally induced by MYOD. In this system, CDR1as promotes goat satellite muscle stem cell differentiation by acting as a ceRNA for miR-7, its main miRNA partner, thereby releasing IGF1R mRNA from miR-7 targeting (Li et al., 2019a).

circRNAs in other tissues

There are also a number of other tissues in which the role of circRNAs has just started to be explored. Below, we highlight just two examples of differentiation processes in which circRNAs have been shown to be differentially expressed and to have a potential role.

circRNAs recently emerged as possible new players in skin development and regeneration as well as in skin diseases such as psoriasis (Kristensen et al., 2018; Liu et al., 2019b). By analyzing RNA-seq data from epidermal stem cells and keratinocytes, circRNAs were found to be highly and differentially expressed along the differentiation process, with a preferential accumulation in keratinocytes with respect to expression in undifferentiated cells (Kristensen et al., 2018). Many of these circRNAs are also modulated independently of their linear counterparts. Interestingly, a significant number of circRNAs that are upregulated independently of their mRNAs are derived from genes related to epidermal growth and differentiation, and a large fraction of them also have a high number of AGO2- and miRNA-binding sites. One particular circRNA that emerged from this group is circZNF91, as it has 24 binding sites for miR23b-3p, a miRNA involved in keratinocyte differentiation, as well as 23 binding sites for miR-766-3p, which is overexpressed in cutaneous squamous cell carcinoma and can target DNMT3B (Kristensen et al., 2018). Therefore, it was suggested that circZNF91 exerts it functions in epidermal differentiation by acting as a miRNA sponge. In this case, the abundance of binding sites for two different miRNAs makes circZNF91 a very interesting ceRNA candidate, which would be worth further investigation.

circRNAs can also contribute to the development of adipose tissue. Recently, circH19 was shown to regulate the differentiation of human ADSCs into adipose cells (Zhu et al., 2020). circH19 is derived from the host gene of the lncRNA H19, which has already been characterized as a regulator of lipid metabolism. circH19 depletion was shown to promote adipogenesis in ADSCs by causing an increase in the expression of lipogenic transcription factors, such as PPARγ (Zhu et al., 2020). circH19 can also bind to the protein PTBP1, which is involved in the cleavage of SREBP1 (also known as SREBF1; which has a fundamental role in lipid metabolism and adipose cell differentiation) and the translocation of its N-terminal cleaved portion from the cytoplasm to the nucleus during adipogenesis. Indeed, it was observed that the knockdown of circH19 enhances the cleavage and translocation of SREBP1 to the nucleus. Therefore, circH19 could inhibit adipogenesis by binding to PTBP1 and counteracting its functions related to SREBP1 processing. The negative role of circH19 in adipogenesis could link it to metabolic syndrome, which is characterized by impaired ADSC differentiation. In fact, high levels of circH19 have been associated with this disorder; circulating circH19, but not the lncRNA, is highly expressed in the serum of patients with metabolic syndrome compared with controls (Zhu et al., 2020).

Discussion

The discovery of a large number and different functions of circRNAs has added further layers of gene expression regulation, increasing our knowledge on the transcriptional potential of eukaryotic cells. Moreover, this information has added a degree of complexity to the comprehension of the structure, function and evolution of eukaryotic genomes.

Despite the continuing growing interest in identifying and characterizing novel circRNA species, there are a few issues that should be considered when approaching circRNA studies. One of them is that even if computational approaches can help to identify bona fide circRNAs, they are not definitive even for very abundant circRNAs. Indeed, validation of the circularity of candidates by independent approaches, such as RNase R treatment to deplete linear molecules or specific RNase H assays to specifically cut the putative circular RNAs followed by RT-PCR or northern blot analysis, are strictly required.

Many aspects of circRNA function, origin and evolution are still awaiting clarification and new methodologies are required to improve these studies. In addition, methodologies to determine the protein and RNA composition of specific circRNA-containing complexes (circRNPs) is strongly needed. RNA pull-down techniques allowing purification of circRNA interactors could be achieved using specific complementary RNA or DNA probes followed by mass spectrometry and/or RNA sequencing. Biotinylated antisense probes have been utilized after crosslinking of cell extracts (Chu et al., 2015; McHugh et al., 2015). However, in order to prevent the co-purification of linear counterparts, either probes specific for the back-splicing junction need to be used or the cell lysate should be pre-treated with RNase R to degrade linear RNAs. The selection of the specific strategy strongly depends on the abundance of the circRNA under study as well as its relative expression level with respect to that of the linear isoform.

Similar attention has to be paid to setting up suitable imaging methodologies that allow the relative quantification and cellular localization of circRNAs versus their linear counterparts. In the last few years, several different enhanced fluorescence *in situ* hybridization methods have been utilized. Among them, the BaseScope Assay has resulted in a highly specific and sensitive strategy (Erben et al., 2018). It is based on the use of two Z-probes designed on the sequences flanking the back-splicing junction that, only when in proximity, can support the next steps of signal amplification (Xu et al., 2018; Harris et al., 2020; Nielsen et al., 2020). Thanks to the amplification step, this procedure enables single-molecule detection with a high signal/background ratio.

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Similar to studies of lncRNAs, phylogenetic analyses coupled with appropriate bioinformatic tools could contribute to the extrapolation of different functional subdomains from the primary sequences of circRNAs. In particular, it would be useful to predict sequences involved in the recognition of other nucleic acids or those acting as scaffolding modules for protein interaction. If we consider that different types of circRNAs may derive from a single primary transcript, it becomes evident that many alternative functions can originate from a single genomic locus that might be subjected to evolutionary selection. The optimization of specific tools for circRNA sequence analysis and prediction of their molecular partners, accompanied by new wet techniques for their validation, will greatly help circRNA research and will hopefully shed light on other relevant roles for this class of transcripts. Recent advances in next-generation sequencing, such as the use of the Nanopore RNA sequencing technology (Oxford Nanopore Technologies, Oxford, UK), will also undoubtedly lead to further progress in the field. Indeed, exact circRNA exon composition cannot be defined by RNA-seq experiments producing short reads, thus limiting circRNA identification to the back-splicing junction. The Nanopore technology allows direct RNA-seq with very long reads to be performed, thereby providing the opportunity for full-length sequencing of circRNAs and therefore for defining their precise sequence (Rahimi et al., 2019 preprint).

Aided by the availability of several prediction tools, the function of a number of circRNAs has been linked to their ability to pair with miRNAs, suggesting that they could act as miRNA sponges by controlling miRNA-circRNA-mRNA networks in different cellular systems. Although in some cases the real proof of this activity has been rigorously provided, in other cases there is no reliable data and confirmations are often based on overexpression experiments. Indeed, one major problem with the sponge theory is the stoichiometry of miRNA molecules versus miRNA-binding sites, as the abundance of miRNAs often happens to be lower than that of the corresponding target sequences. However, as suggested in some cases, specific subcellular compartments can be created where miRNAs and target sequences can be tethered together, thus overcoming the concentration issue.

Although many different molecular mechanisms are starting to be attributed to circRNAs in *in vitro* cellular systems, there is an urgent need for appropriate *in vivo* model systems that can be used to gain a deeper understanding of circRNA function in development and cell differentiation (see Box 1). However, owing to their intertwined expression with their linear counterpart, the design of suitable knock-out strategies that prevent circRNA formation without affecting the mRNA should not be overlooked. When intronic sequences responsible for circularization are known, such as the case of Alu sequences, the deletion of the reverse complementary sequence in the downstream flanking intron can effectively lead to circRNA depletion, as recently described in an *in vivo* mouse knockout model for cia-cGAS (Xia et al., 2018). One additional strategy is to precisely delete one of the introns flanking the exon(s) that undergo circularization; however, since a common feature of such introns is that they are very long, this approach can be challenging and side effects on the expression levels of the linear counterpart have to be checked. As a further strategy, when siRNAs highly specific for the back-splicing junction are available, it is possible to envisage their targeted expression *in vivo* as applied in *Drosophila* for the knockdown of the most abundant fly circRNA circMbl (Pamudurti et al., 2018 preprint).

Finally, understanding the regulation of circRNA expression, and its interdependence with its linear counterpart, is an important topic that has so far been poorly addressed. The main problems are related to understanding how the choice between back and linear splicing is controlled and to deciphering the factors that control circRNA subcellular localization. Moreover, it will be important to understand whether any functional interconnection between the circular and linear forms originating from the same genomic locus exists and how this contributes to the evolution and complexity of gene expression regulation.

In conclusion, circRNAs are much more abundant than previously thought and they exhibit clear cell type specificity. The regulation of this important class of RNAs, and the *cis*- and *trans*-acting factors that regulate their homeostasis in the cell, are important issues that need to be studied in depth and with appropriate technologies in the future.

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Competing interests

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