UNIVERSIDADE DO ALGARVE

IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF BONE-RELATED MICRORNAS: FUNCTIONAL IMPLICATIONS

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Our genes define who we are.
By improving our knowledge about DNA/RNA,
we can impact on our lives.

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CONTENTS

Abstract

MicroRNAs (miRNAs) are a conserved class of small RNAs providing a post-transcriptional mechanism for fine-tuning of intricate physiological and pathological cellular processes, such as those affecting development. Skeletogenesis however, was so far poorly investigated and mainly focused on mammalian models, with a general lack of knowledge concerning other vertebrates. We aimed at the identification of bone-related miRNAs and their characterization from an evolutionary perspective, using fish (mostly zebrafish) as model, in comparison to mammalian systems.

First, we focused on miR-223, a miRNA that was associated with bone remodelling. We demonstrated that miR-223 genomic organization/context and primary/secondary structures are largely maintained between human and zebrafish. As in mammals, miR-223 expression in zebrafish was highly correlated with hematopoietic events and osteoclastogenesis. Finally, miR-223 targets identified in mammals were also predicted in zebrafish, supporting a functional conservation of this miRNA.

In a second set of experiments, we studied the biological role of miR-29a, a bone-related miRNA that was fairly investigated in mammals, but with no mineralogenic effects yet demonstrated. We took advantage of our fish bone-derived systems to explore miR-29a mineralogenic effects through gain-of-function experiments. We demonstrated a strong stimulation of this process through a mechanism probably involving the canonical Wnt signalling. Once more, through bioinformatics analysis, patterns of expression and target prediction/validation, we provided evidences for miR-29 conservation throughout evolution.

Finally, we explored miR-214 putative roles on skeleton formation in vertebrates. Although our initial hypothesis of miR-214 involvement in osteogenesis was recently demonstrated by Wang et al. (2013), we proceeded with our investigation and finally showed that miR-214 is also associated with chondrogenesis. Overexpression of miR-214 in ATDC5 cells mitigated differentiation and down-regulated Mgp and Osteocalcin, probably by targeting Atf4.

This work provides novel evidence that some miRNAs have conserved functions across vertebrates and, probably, conserved regulatory mechanisms of action.

Keywords: miRNAs; conservation; zebrafish; regulation; expression; bone.

Resumo

Nos últimos anos, assistiu-se a uma marcante expansão na área da biologia molecular, devendo-se isto principalmente à descoberta de pequenas moléculas de RNA não codante e ao seu modo peculiar de intervir na regulação genética. Dentro deste grupo de moléculas, os microRNAs (miRNAs) são, definitivamente, a classe melhor compreendida, o que se comprova pelo crescimento exponencial do número de trabalhos publicados desde a sua descoberta. Os miRNAs, na sua forma matura, são RNAs com aproximadamente 22 nucleótidos (nt), altamente conservados em vertebrados e que asseguram um controlo apertado de vários processos celulares através de uma regulação pós-transcricional. Esta regulação ocorre através da ligação específica do miRNA à 3'UTR do RNA mensageiro (mRNA). Neste mecanismo, destaca-se o envolvimento do complexo RISC (RNA-induced silencing complex; associado ao miRNA), a complementaridade da denominada região "seed" (extremidade 5' do miRNA) ao mRNA, e o consequente bloqueio da tradução ou degradação do mRNA. Desta forma, cada miRNA pode regular centenas de genes transcritos, e de facto, hoje em dia pensa-se que a maioria dos genes humanos são controlados por miRNAs. Assim, os miRNAs são considerados não só importantes reguladores de múltiplos processos biológicos, incluindo desenvolvimento, diferenciação e apoptose celular, mas também responsáveis por vários processos patológicos, como o cancro, onde se observou que inúmeros miRNAs têm a sua expressão desregulada. Assim, a caracterização dos miRNAs (a vários níveis) é fundamental para a compreensão das suas funções, permitindo alargar também o conhecimento dos processos biológicos e patológicos onde estão envolvidos.

Apesar do conhecimento sobre miRNAs ter aumentado francamente nos últimos anos, o papel dos miRNAs na formação e homeostasia do osso ainda está pouco caracterizado, e a maioria dos estudos tem abordado principalmente esta forma de regulação em mamíferos, havendo assim uma lacuna de conhecimento na regulação destes processos noutros vertebrados. Neste sentido, este trabalho focou-se na identificação de miRNAs potencialmente envolvidos na regulação do osso e na sua caracterização numa

perspectiva evolutiva, usando o peixe (essencialmente o peixe-zebra) como modelo, e em comparação com mamíferos.

Numa primeira abordagem, focámos a nossa investigação no estudo do miR-223, um miRNA anteriormente associado à diferenciação celular da linhagem hematopoiética e ao processo de remodelação óssea. Neste estudo, demonstramos que a organização e contexto genómicos do miR-223 estão preservados em vertebrados, verificando-se uma conservação das estruturas primária e secundária do pre-miR-223 em 46 espécies. Este estudo mostra ainda que a expressão deste miRNA se correlaciona com determinadas fases do desenvolvimento do peixe-zebra onde a hematopoiese e a osteoclastogénese são eventos predominantes. Além disso, este estudo mostra que o miR-223 apresenta uma expressão elevada no principal órgão hematopoético de peixes e ratinhos adultos (rim anterior e medula óssea, respectivamente), sugerindo que a função hematopoiética também se encontra conservada. Por último, através de análise bioinformática demonstrámos que a regulação de genes alvo do miR-223 em mamíferos também deverá estar mantida em peixe-zebra.

Na secção seguinte estudámos o papel biológico do miR-29a, cujo efeito osteogénico em mamíferos se encontra bem caracterizado, mas sem nenhum fenótipo mineralogénico ainda associado. Neste estudo utilizámos uma linha celular derivada do osso de peixe previamente desenvolvida no nosso laboratório e com capacidade de mineralização *in vitro*. A fim de explorar os efeitos mineralogénicos do miR-29a foram realizadas experiências de ganho de função. O aumento dos níveis endógenos deste miRNA resultaram num incremento da mineralização da matriz extra-celular, o que provavelmente terá sido devido a uma aceleração da diferenciação celular pelo potenciamento da via de sinalização Wnt, tal como evidenciado pela acumulação de um dos seus principais componentes, a β-catenina. Além disso, foi demonstrada a conservação da função deste miRNA através de estudos baseados em homologia de sequências, análise de sintenia, padrão de expressão tecidular e na manutenção da regulação do SPARC, um alvo previamente descrito em mamíferos. Reforçou-se assim a ideia de que o miR-29a é um regulador crucial na diferenciação de osteoblastos, induzindo um aumento da mineralização em sistemas *in vitro*.

Finalmente, explorámos a hipótese do miR-214 ser regulador da formação do esqueleto/osso, em vertebrados. Apesar da nossa primeira hipótese, que consistia no envolvimento do miR-214 na osteogénese, ter sido entretanto demonstrada através do trabalho realizado por Wang et al. (2013), continuámos com este estudo, tentando demonstrar um potencial envolvimento deste miRNA na condrogénese, um processo essencial na formação do esqueleto de vertebrados. Através do padrão de expressão espacial e temporal do miR-214 durante o desenvolvimento do peixe-zebra, verificou-se uma clara associação com estruturas cartilagíneas. Adicionalmente, demonstrámos que a região reguladora (promotor) do transcrito primário deste miRNA se encontra conservada em oito vertebrados, assim como os locais de ligação de factores de transcrição (associados à condrogénese e/ou osteogénese) identificados. De acordo com a análise funcional deste promotor, concluiu-se que esta região reguladora (quer de peixe-zebra quer de humano) é activada e regulada de forma semelhante em condrócitos e osteoblastos. Por último, verificou-se que a sobreexpressão do miR-214 nas células ATDC5, um modelo *in vitro* para a condrogénese, atenua a diferenciação condrocítica, possivelmente através da regulação do gene Atf4. O decréscimo simultâneo de dois marcadores ósseos, a Mgp e a osteocalcina, aquando da sobreexpressão deste miRNA sugere que a mineralização dos condrócitos poderá estar comprometida nesta condição. Assim, propomos que o miR-214 desempenha um papel fundamental na formação do esqueleto de vertebrados, não apenas pela regulação da osteogénese, mas também pelo controlo da condrogénese, promovendo assim a normal e equilibrada formação de estruturas ósseas e cartilagíneas.

No seu conjunto, estes estudos evidenciam uma conservação na função e mecanismos de regulação de muitos dos miRNAs identificados em vertebrados. Este conhecimento é bastante importante, por exemplo para a investigação de tratamento de patologias, uma vez que permite a utilização de modelos alternativos no rastreio de potenciais alvos terapêuticos, com particular destaque para as vias reguladas por miRNAs. Nesta perspectiva, em doenças como por exemplo a osteoporose, onde se verifica uma perda de massa óssea, terapias que estimulem a acção de miRNAs que promovam a osteoblastogénese ou que inibam a osteoclástogénese, são atractivas e com

grande potencial na estimulação da formação óssea ou na redução da reabsorção óssea excessiva, respectivamente.

Palavras-chave: miRNAs; conservação; peixe-zebra; regulação; expressão; osso.

List of abbreviations

Ago2 - Argonaute 2
AP2alpha - transcription factor AP-2 alpha
Atf4 - activating transcription factor 4
BMP - bone morphogenetic protein
BS - binding site
Col10a1 - collagen type X alpha 1
Col2a1 - collagen type II alpha 1
DGCR8 - DiGeorge syndrome critical region in gene 8
dpf - days post fertilization
dsRNA - double-stranded RNA
ECM – Extracellular matrix
GFP - green fluorescent protein
hpf - hours post fertilization
ISH - in situ hybridization
kb – kilobase pairs
Mgp - Matrix Gla Protein
miRNA – microRNA
mRNA - messenger RNA
MSCs - mesenchymal stem cells
ncRNA - non-coding RNA
nt - nucleotide
Oc – Osteocalcin
Osx or Sp7 - Osterix
Pol II - RNA Polymerase II
pre-miRNA - miRNA precursor
pri-miRNA – primary microRNA transcripts
qPCR - real time-PCR
RACE - rapid amplification of cDNA ends
RISC – RNA induced silencing complex
RNAi - RNA interference
SP1 - specific protein 1 transcription factor
ssRNA - single-stranded antisense RNA
TFBS - transcription factor binding site
TFs - transcription factors
TNAP - alkaline phosphatase
U6 – U6 small nuclear RNA
UTR - untranslated region
WT - wild type

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CHAPTER 1

General Introduction

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1.1. MicroRNAs

1.1.1. Overview

In the last years, only few research areas of biology have witnessed such a remarkable expansion as that observed for RNA molecular biology. Although this breakthrough has occurred through many fronts, one of the areas where major progresses were obtained concerned the discovery of the mode of action and impact of small non-coding RNAs (ncRNAs) on the regulation of genes and genomes. Since the first discovery of double-stranded RNA (dsRNA) ability to regulate gene expression by antisense base-pairing to target messenger RNA (mRNA), a process named RNA interference (RNAi) (Fire et al., 1998), an enormous increase in the number of identified small ncRNAs was observed, and these have been found in animals, plants, fungi and viruses. Despite the existence of various classes of small ncRNAs, these molecules are generally divided in three main categories based on their biogenesis, structure, associated effector proteins and biological functions (Bartel, 2009; Moazed, 2009): (i) short interfering RNAs (siRNAs), which are processed by Dicer from long dsRNAs into duplexes of 21-25 nucleotides (nt) in length and act through the RNAi pathway to regulate gene expression (Ambros et al., 2003; Reinhart and Bartel, 2002); (ii) piwi-interacting RNAs (piRNAs) which are longer RNAs of about 25-30 nt that interact with Piwi proteins (Aravin et al., 2007); and (iii) microRNAs (miRNAs) (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001; Lee et al., 1993), which will be described in detail in the next sections. MiRNAs are the best understood of these three classes, and the emergent perception of their importance led to a boost on publications in the past years with over 32500 scientific reports currently recorded and from them more than 30000 were published during the course of the work here presented (last checked on the 23 of June 2014 at PubMed database, http://www.ncbi.nlm.nih.gov/pubmed) (Figure 1.1).

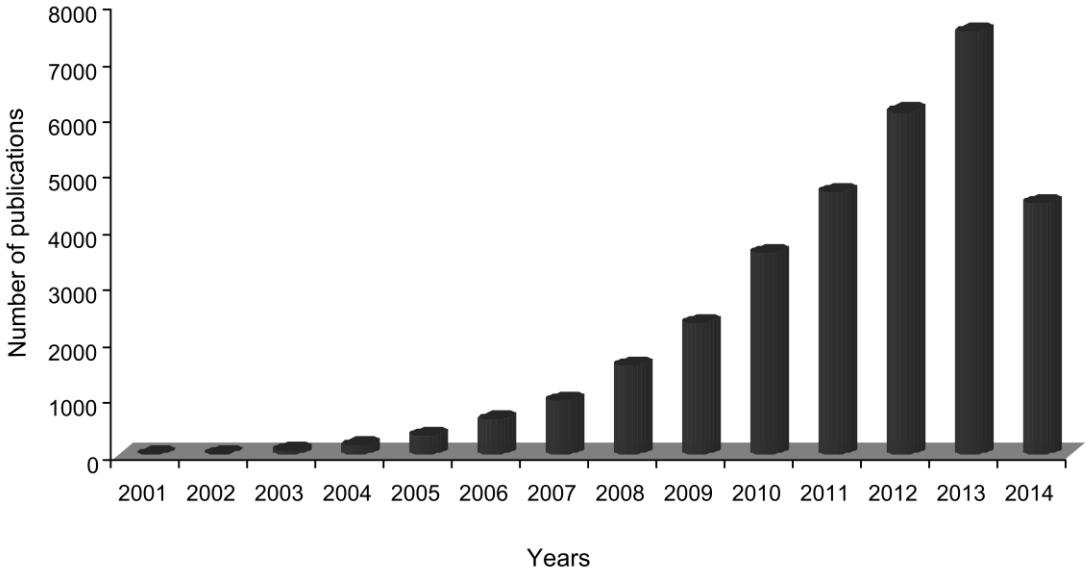

Figure 1.1. Graphical representation of the number of publications in the PubMed database per year. The keywords microRNA, microRNAs and miRNA were searched in the title and/or abstract of articles available. Note that for 2014, the number of publications refers to publications until the 23 of June 2014.

A perspective on animal miRNA discovery, genomics, biogenesis, mechanisms and functions will be described in the following sections.

1.1.2. History of miRNAs

The central dogma of molecular biology which considered that RNA molecules acted as simple messengers between DNA, encoding cellular instructions, and proteins, the end-products which executed those instructions (Crick, 1970), started to be questioned when researchers realized that ncRNA molecules could interfere with gene expression. The biological phenomenon of antisense control mechanisms was first recognized in the late 70s and early 80s when scientists found that exogenous oligonucleotides with a complementary sequence to ribosomal RNA could prevent ribosome function in *Echerichia coli* (Eckhardt and Lührmann, 1979; Jayaraman et al., 1981). More then ten years later, Ambros and colleagues discovered that *lin*-4, a gene known to be essential for developmental timing of the nematode worm *Caenorhabditis elegans* larvae, did not encode a protein, but rather produced a pair of small RNAs, one containing 22 nt and another containing ~61 nt sequence with a predicted stem loop structure and proposed to be the precursor of the shorter (~22 nt) molecule (Lee et al., 1993). Both RNAs were found to have antisense

complementarity with multiple sites in the 3'-untranslated region (UTR) of *lin*-14 transcript (Lee et al., 1993; Wightman et al., 1993). Based on this information, *lin-4* putative binding and regulation of *lin-14* was proposed (Wightman et al., 1991). However, it was only in 1993 that this mechanism was demonstrated, when *lin*-14 transcript levels were shown to be constant throughout development of *C. elegans*, whereas LIN-14 protein levels were not, indicating a post-transcriptional regulation (Wightman et al., 1993). The authors demonstrated that: (i) the post-transcriptional regulation of *lin-14* by *lin-4* generated a temporal gradient of Lin-14 protein during *C. elegans* development; (ii) *lin-14* 3'UTR was essential and sufficient for *lin-4*-mediated temporal regulation; and that (iii) multiple conserved elements in the *lin-14* 3'UTR were complementary to, at least, a core of 7 nt in the 5´-end of *lin-4*, mediating part of the temporal gradient activity of the *lin-14* 3´-UTR (Wightman et al., 1993). This regulatory process was later proven to be essential for worms to proceed from their first larval stage to the second, as reviewed by Rougvie (2005). It took seven years to discover that this mechanism was not an isolated event, until a second small regulatory RNA, let-7, was identified (Reinhart et al., 2000). Similarly to *lin-4*, *let-7* was shown to operate by specific binding to the 3'UTR and repression of *lin*-41 and *hbl*-1 mRNAs (Lin et al., 2003; Reinhart et al., 2000; Slack et al., 2000; Vella et al., 2004). By then, *let-7* was found to be highly conserved throughout metazoan (Pasquinelli et al., 2000), contradicting the general idea that *lin-4* and *let-7* were a worm-specific peculiarity. Meanwhile, dsRNA mediated gene down-regulation in *C. elegans* was reported to be far more potent than single-stranded antisense RNA (ssRNA) (Fire et al., 1998), which brought new insights into the putative mechanisms of RNA interference (RNAi), as it will be described next in detail. These findings propelled intense genome-wide searches to identify additional endogenous small regulatory RNAs, which ended to be demonstrated in 2001 (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). This finally led to the recognition that microRNAs (miRNAs) represent a distinct, conserved and abundant class of regulatory genes. The importance of non-coding RNA was further supported when the draft of the human genome project was concluded, and revealed that the extent of protein-coding genes covers only about 2% of the human genome (Lander et al., 2001). Remarkably, while the number and

size of protein-coding genes was shown to not vary substantially with increasing developmental complexity, this was not the case for non–protein coding sequences in genomes, indicating that these sequences may enclose increasingly intricate regulatory information (Taft et al., 2007).

Nowadays, RNA molecules are known to function not only as messengers of protein production, but also as key features of the gene regulatory networks with 30424 mature miRNAs identified so far in animals, plants, algae, amoeba, diatom and viruses, and deposited in the miRNA database (miRBase Release 20, http://www.mirbase.org/). MiRNAs are now known to be involved in a variety of biological processes, including cell proliferation and differentiation (Yang et al., 2011d), apoptosis (Li et al., 2011a), organogenesis (Giraldez et al., 2005; Papaioannou et al., 2013), and also in pathological processes, such as cancer (Plaisier et al., 2012; Wang et al., 2013b) and infection / inflammation / immunity (Baltimore et al., 2008; Haneklaus et al., 2013). Outstandingly, bioinformatics predictions suggest that miRNAs control up to 60% of all human protein-coding genes (Friedman et al., 2009), further confirming an essential role in eukaryotic gene regulation.

1.1.3. miRNA Genes

Our knowledge about miRNA biology has been significantly increased following the discovery of *let-7*. Most of the studies, however, have been focusing on processing and targeting by miRNAs. In this regard, despite being important regulatory steps in miRNA biogenesis, miRNA genomics and transcription regulation are still poorly understood. MiRNAs are endogenous small non-coding RNAs (~22 nt) generated from conserved hairpin structures that are transcribed from diverse regions of the genome as long primary transcripts (pri-miRNAs), scattered in all chromosomes from humans to zebrafish (Bartel, 2004; Kim et al., 2005; Pillai, 2005; Thatcher et al., 2008). The first miRNAs identified, *lin-4* and *let-7*, were shown to be located in non-coding regions in-between genes and transcribed from unidentified promoters, leading to the initial thought that most miRNA genes were located in intergenic regions (Lagos-Quintana et al., 2001; Lau et al., 2001) and thus named intergenic miRNAs (Fig. 1.2a). However, the identification of several intronic miRNAs in *C.*

elegans, mouse an human genomes (Ambros et al., 2003; Rodriguez et al., 2004) and the demonstration of gene silencing mechanisms associated to miRNAs derived from introns (Ying and Lin, 2004), evidenced a new miRNA category: intronic miRNAs (Fig. 1.2 b). In fact, new technology and refined mapping demonstrated that the vast majority of mammalian miRNAs reside within the intronic regions of either protein-coding genes or non-coding transcripts (Griffiths-Jones et al., 2008; Rodriguez et al., 2004). Although this estimation varies between species, the location of several intronic miRNAs is quite conserved among different organisms (Kim and Nam, 2006). Intronic miRNAs are generally sense orientated with their host gene and expression of both miRNA and host gene largely coincides, suggesting a co-regulation and generation from a common precursor transcript (Baskerville and Bartel, 2005).

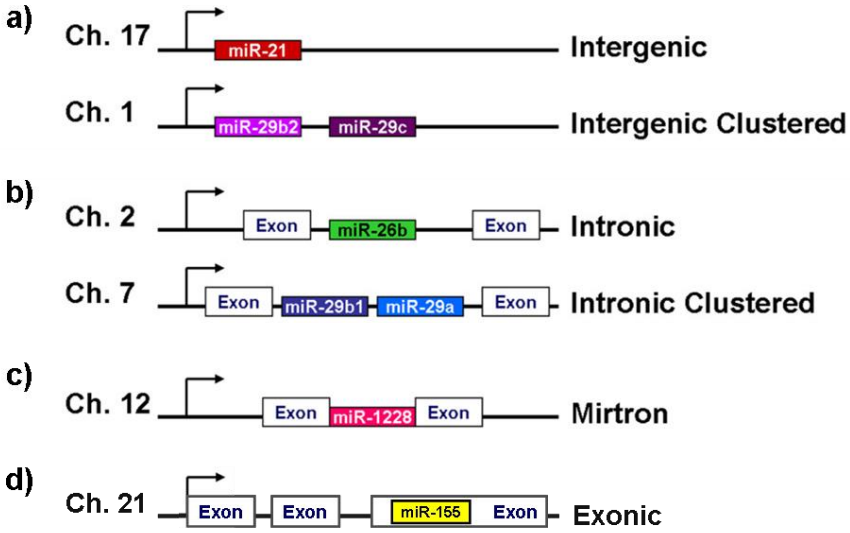

Figure 1.2. Genomic organization of miRNA genes. miRNA genes can reside (a) in-between genes, named intergenic miRNAs (alone or clustered); (b) in the intron of ncRNA or protein-coding genes (alone or clustered), called intronic miRNAs; (c) in a short intron, the mirtrons; or (d) in the exon of ncRNAs, which are called exonic miRNAs. *Adapted from Kapinas and Delany (2011)*.

A few miRNA precursors, called 'mirtrons', comprise the full intron size and, when spliced, are able to bypass the first cleavage step in miRNA processing (Berezikov et al., 2007) (Fig. 1.2 c). Also, a small subset of miRNAs, approximately 10%, is located within exons of non-coding genes (Kim et al., 2009; Rodriguez et al., 2004) (Fig. 1.2 d). In addition, 36% to 47% of known miRNAs are found in clusters and might be transcribed as single polycistronic

primary transcripts in vertebrates (Griffiths-Jones et al., 2008; Olena and Patton, 2010; Thatcher et al., 2008) (Fig. 1.2 a, b). Clustered miRNAs are frequently related to each other in sequence, suggesting that miRNA clusters might be a consequence of gene duplication. Exceptionally, some clusters contain representatives of different miRNAs families without apparent sequence homology (Kim and Nam, 2006). A possible explanation for this relies on the ability for clustered miRNAs to target the same gene or different genes in the same pathway (Yuan et al., 2009b).

1.1.4. Identification of miRNAs

Nowadays, both biological and bioinformatics approaches for miRNA identification have yielded many thousands of miRNA sequences and novel miRNAs still appear almost on a daily basis. Identified miRNAs are deposited in miRBase (www.mirbase.org/) (Kozomara and Griffiths-Jones, 2011), a widely known public database for published miRNA sequences and respective annotation (miRBase database), and also for new miRNA genes prior to their publication (miRBase Registry). Each miRBase entry matches a predicted hairpin fraction of a miRNA transcript and contains information on the location and sequence of the mature miRNA, its genomic location, target prediction, conservation and experimental validation (Griffiths-Jones et al., 2008; Kozomara and Griffiths-Jones, 2011). The current version of miRBase (Release 20, updated in June 2013) contains 24521 entries, which express 30424 mature miRNAs in 206 different species.

The first miRNAs were identified by forward genetic screens (Lee et al., 1993; Reinhart et al., 2000). Directional cloning can be applied in every organism, which can be an advantage when the available genomic information is scarce or non-existent. Combined cloning and bioinformatics approaches proved to be particularly valuable in the first years of miRNA research. Nevertheless, with the recent advances in next-generation sequencing, deep sequencing has been applied to both miRNA discovery and quantification in several organisms (Bizuayehu et al., 2013; Castellano and Stebbing, 2013; Wei et al., 2012). In fact, this technology boosted the number of miRNAs identified in different species, further increasing the challenge of functional annotation and

driving a considerable advance in bioinformatics approaches for miRNA target prediction and systems-based analysis of miRNA function.

1.1.5. Biogenesis of miRNAs

1.1.5.1. miRNA Transcription

Characterization of miRNAs genomic organization, transcription and regulation is still ongoing. MiRNAs transcription is known to be mostly mediated by RNA polymerase II (Pol II), with primary transcripts (pri-miRNAs) bearing Pol II signatures such as a 7-methyl guanylate cap at the 5' end and poly (A) tail at the 3' end (Cai et al., 2004; Davis and Hata, 2009; Ozsolak et al., 2008). RNA Pol III has also been found to be involved in the transcription of some miRNAs, but this mechanism has been rarely observed (Borchert et al., 2006; Ozsolak et al., 2008). Supporting this, a large scale analysis of intergenic miRNAs structures and mapping of human miRNA promoters (involving chromatin immunoprecipitation) indicated that miRNA promoters display all features commonly associated with Pol II-mediated transcription, such as CpG islands, TATA boxes, transcription factor IIB recognition sites, initiator elements and histone modifications (Corcoran et al., 2009; Ozsolak et al., 2008; Saini et al., 2007). Besides having dedicated promoters, miRNAs were demonstrated to be first transcribed as long pri-miRNAs, with 3-4 kb (kilobase pairs) in length (Gu et al., 2006; Ozsolak et al., 2008) and containing transcript start sites (TSSs) and poly(A) signals located within approximately 2 kb upstream and downstream of the miRNA precursor (pre-miRNA), respectively (Fig. 1.3) (Saini et al., 2007). Ozsolak and colleagues also demonstrated that about one third of intronic miRNAs have their own promoter regions, enabling a different regulation from the host gene (Ozsolak et al., 2008). Furthermore, coupling between transcription and processing has been verified for both intergenic and intronic miRNAs. An important difference however, relies on the fact that intergenic miRNAs co-transcriptional processing is coupled with termination (Ballarino et al., 2009), while intronic miRNAs processing seems to occur co-transcriptionally in cooperation or preceding splicing of the primary transcript (Janas et al., 2011; Kim and Kim, 2007; Morlando et al., 2008).

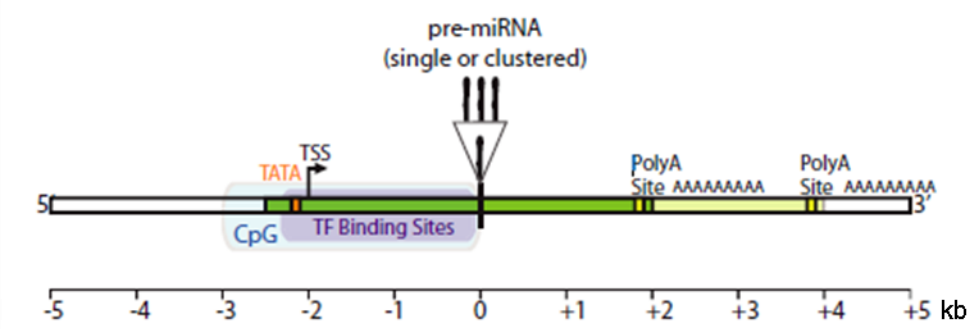

Figure 1.3. Representation of a canonical intergenic pri-miRNA. Intergenic pri-miRNAs (green) have dedicated promoters with TATA boxes, transcriptional start sites (TSS), CpG islands (CpG) and transcription factor (TF) binding sites. Pri-miRNAs transcripts can contain one or several pre-miRNAs and more then one polyadenylation (Poly A) sites. *Adapted from Saini et al. (2007)*.

Regarding transcriptional regulation, mRNAs and miRNAs also present important similarities. Indeed, the network of transcription factors (TFs) that control protein coding genes is also found to regulate miRNA transcription, orchestrating cell-fate decisions, cell differentiation and tissue and developmental stage-specificity (Schmeier et al., 2009). In addition, as in protein coding genes, TF-binding sites (TFBS) are 8-15 base pairs (bp) long and are generally located nearby TSSs, close to the pre-miRNA. Furthermore, 60% of human miRNAs have clustered TFBS preferentially located within a 1-kb region (Saini et al., 2007). Interestingly, emerging evidence indicates that miRNAs have a common tendency to regulate transcription factors that drive their expression, cooperating in complex regulatory networks through feedback loops to regulate cell decisions (Fazi et al., 2005).

In summary, regulation of pri-miRNA transcription is one of the most important features controlling miRNA abundance and a full understanding of that process requires a comprehensive characterization of the genomic location and extent of pri-miRNAs, including TSSs, promoters and TFBS.

1.1.5.2. miRNA Processing

As mentioned before, transcription of miRNA genes by RNA Pol II originates long, capped and polyadenylated pri-miRNAs which can contain one or more miRNAs (Cai et al., 2004). Once transcribed, pri-miRNAs fold into imperfectly base-paired stem-loop (also known as hairpin) structures that are

further processed. In animals, maturation of miRNAs occurs in two processing steps, each one catalyzed by a ribonuclease III (RNase III) endonuclease in cooperation with a double-stranded RNA-binding domain (DsRBD) protein. This process is summarized in Fig. 1.4 and will be described next.

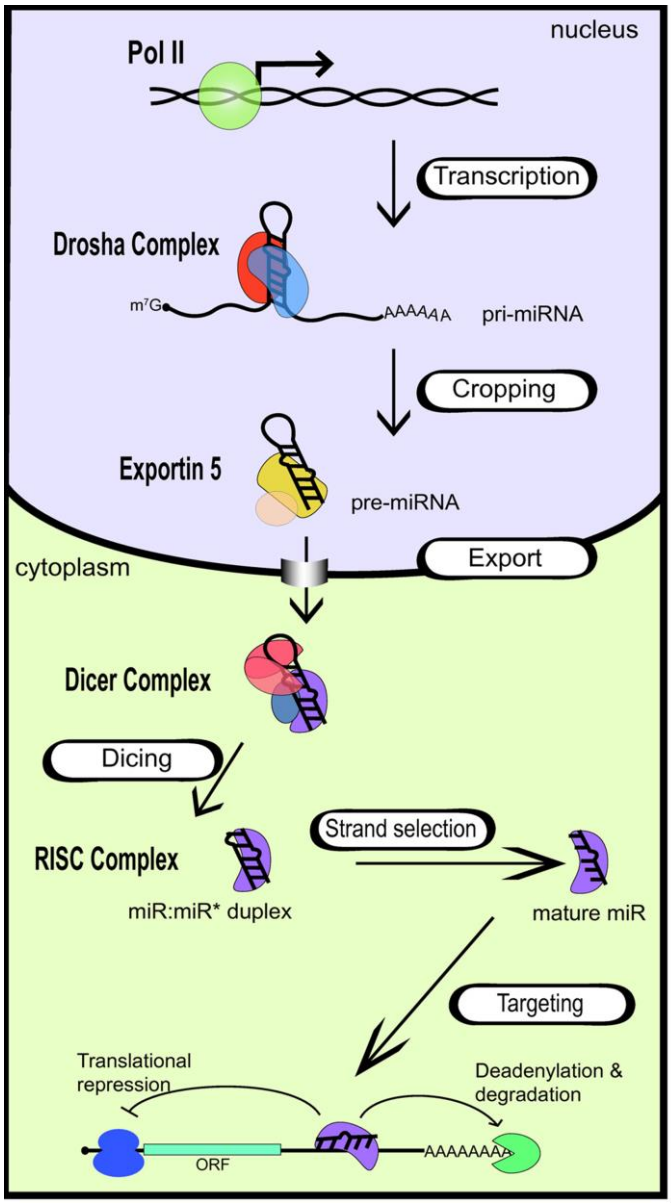

Figure 1.4. Biogenesis of miRNAs. Transcription of miRNA genes by RNA Poll I originates capped and polyadenylated transcripts that fold into hairpin structures (pri-miRNAs). Cleavage by Drosha in the nucleus generates a smaller miRNA precursor (pre-miRNA) which is then exported into the cytoplasm and further processed by Dicer to generate a miRNA:miRNA* duplex. Once this duplex is assembled into RISC, the miRNA* is discarded, and the mature miRNA guides this complex to the target mRNA. Translation inhibition or mRNA degradation occurs by binding of the miRNA to the 3'UTR of target mRNA. *In Davis and Hata (2009).*

The first step of miRNAs processing is catalysed by the nuclear RNase III Drosha, which cleaves the stem-loop structures in pri-miRNAs to originate a ~70 nt pre-miRNA (Han et al., 2004; Lee et al., 2002, 2003, 2006). The accuracy and efficiency of this process is assured by the cooperation of DiGeorge syndrome critical region in gene 8 (DGCR8) (known as Pasha in

Drosophila and *C. elegans*), which interacts with Drosha forming a pri-miRNA processing complex named the Microprocessor complex (Faller et al., 2010; Han et al., 2004, 2006). Efficient processing by Drosha requires (i) an hairpin with a large terminal loop (≥ 10 nt); (ii) two helix turns (~22 nt) that encode the miRNA:miRNA* duplex plus (iii) one helix turn (~11 nt) of the lower stem (Han et al., 2006; Zeng et al., 2005b) (see Fig. 1.5). Cleavage by Drosha occurs approximately 11 nt away from the ssRNA-stem loop junction, defining one end of the mature RNA. The resulting pre-miRNA has a 5' phosphate group and a 2-3 nt 3' overhang characteristic of RNase II cleavage of dsRNA (Han et al., 2004, 2006; Zeng et al., 2005b).

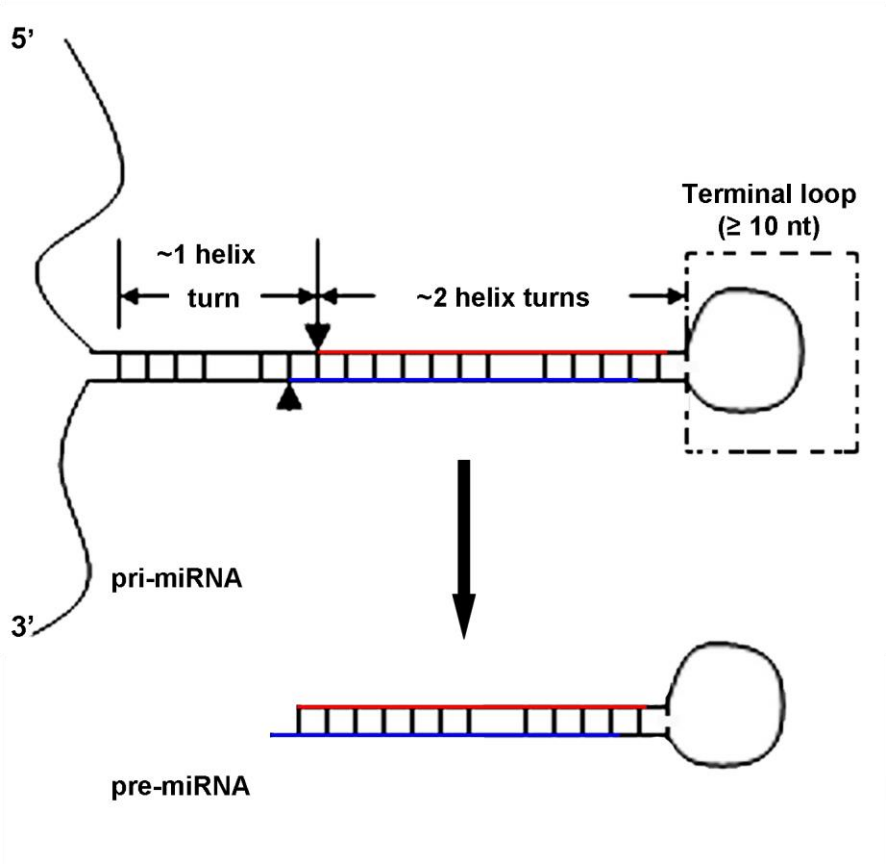

Figure 1.5. Representation of pri-miRNAs structure and Drosha cleavage. Pri-miRNAs fold into stem-loop structures with a central stem of approximately 33 nt (3 helix turns). The lower stem consists of one helix turn, flanked by ssRNA, and the upper stem (which encodes the miRNA duplex) comprises 2 helix turns, flanked by a terminal loop. Drosha cleavage sites are indicated in the pri-miRNA by verticals arrowhead and arrow. The miRNA duplex is represented by red and blue lines in the upper stem. *Adapted from* Zeng et al. (2005b).

After Drosha processing, Exportin-5 (*XPO5*), a nuclear export factor, recognizes the characteristic end structure of pre-miRNAs and exports it to cytoplasm in a Ran-GTP (RAs-related Nuclear protein) dependent manner,

through nuclear pore complexes (Bohnsack et al., 2004; Lund and Dahlberg, 2006; Lund et al., 2004). In the cytoplasm, the pre-miRNA is further processed by another highly conserved RNase III, Dicer, together with its dsRBD partners, which are apparently required for miRNA stability and effector complex formation (Lee et al., 2013; Pilotte et al., 2011). Then, Dicer cleaves the pre-miRNA at the terminal loop, liberating a ~22 nt-long RNA duplex (Bartel, 2004; Macrae et al., 2006; Pillai, 2005). This miRNA duplex contains the mature miRNA and the miRNA* (which is by definition the small RNA in the opposite side of the pre-miRNA stem loop), which are partially paired due to the 5' and 3' overhangs resulting from both Drosha and Dicer cleavages. Evidences collected in the last few years indicate that Dicer processing involves the binding of TRBP (trans-activator RNA (*tar*)-binding protein) to the miRNA duplex and, after cleavage, TRBP recruits Argonaute 2 (Ago2). Ago2 along with Dicer contribute to the assembly of RISC (RNA induced silencing complex) forming the RISC loading complex (RLC) (Chendrimada et al., 2005; Wahid et al., 2010). Once the miRNA duplex is loaded into Ago protein of RISC, the RNA strand with the lowest thermodynamic stability at its 5'-end (called guide strand) remains bounded to this complex while the miRNA* (passenger strand) is degraded (Schwarz et al., 2003). In some cases miRNAs* can be loaded into RISC and originate functional miRNAs. The precise mechanism through which the RNA loading into Ago occurs is still not understood. Concerning Ago2, this is the only one of the four Ago proteins in humans that is known to have endonucleolytic activity, being widely described as the RISC slicer (Song et al., 2004). However, Ago2 is also known to participate in the removal of miRNA passenger strand (Diederichs and Haber, 2007). Interestingly, all four Ago proteins are known to enhance production or stability of mature miRNAs (Diederichs and Haber, 2007). Finally, the mature miRNA guides the RISC to its target transcript, leading to its degradation or translation repression.

1.1.6. miRNA Mechanism of action

The first identified miRNA, lin-4, was shown to down-regulate the protein levels of LIN-14 (Lee et al., 1993). Five years later, Mello's group showed that dsRNA was far more effective in inhibiting the expression of specific mRNA

than ssRNA, uncovering the phenomena of RNAi (Fire et al., 1998). Although at that time these authors speculated that the inhibition process was based on a catalytic mechanism (Fire et al., 1998), miRNAs mode of action was later demonstrated to rely mostly on translation inhibition. However, the mechanism by which miRNAs regulate gene expression is still under debate. Indeed, different studies have demonstrated that down-regulation of protein levels can occur by either inhibition of translation initiation or elongation, premature termination of translation or co-translational inhibition (Eulalio et al., 2008). Additionally, miRNAs can induce target mRNA degradation (Schmitter et al., 2006) and also sequester mRNAs into cytoplasmatic foci called P-bodies, for storage or degradation (Castilla-Llorente et al., 2012) (Fig. 1.6). Recent mechanistic models proposed that miRNA-mediated gene silencing might occur by successive steps, combining translation inhibition and mRNA degradation (Béthune et al., 2012; Djuranovic et al., 2011).

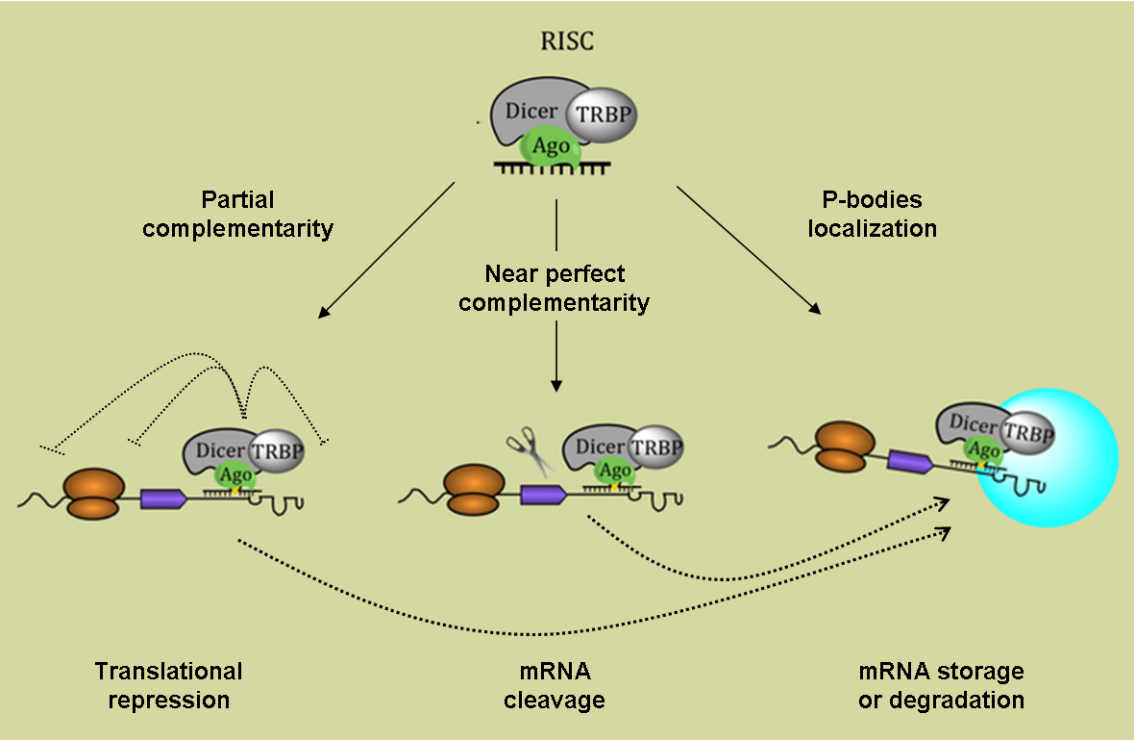

Figure 1.6. Putative mechanisms for miRNA-mediated post-transcriptional regulation. Translational repression occurs when miRNAs bind to their target mRNAs by partial complementarity. Protein production can be blocked by interference with the initiation, elongation or termination steps. A near perfect base pairing between miRNA and target mRNA can originate cleavage of the target mRNAs leading to mRNA decay. Both translation repression and mRNA cleavage can occur in P-bodies, where storage or degradation of mRNAs occurs. *Adapted from Pedroza-Torres et al. (2014)*.

Despite the different mechanisms for miRNAs regulation of gene expression, a common feature was generally shown to be associated to this process, i.e. miRNAs binding to the 3'UTR of target mRNAs through imperfect complementarity. In few exceptional cases however, this regulation involves binding to the 5'UTR or to the coding sequencing of mRNA targets (Duursma et al., 2008; Ørom et al., 2008). The complementarity between miRNAs and mRNA targets is generally confined to the 5' region (nucleotides 2-8) of the miRNA, which has been named the 'seed region', illustrating its contribution to target mRNA binding (Lewis et al., 2003a, 2005; Pillai, 2005) (Fig. 1.7).

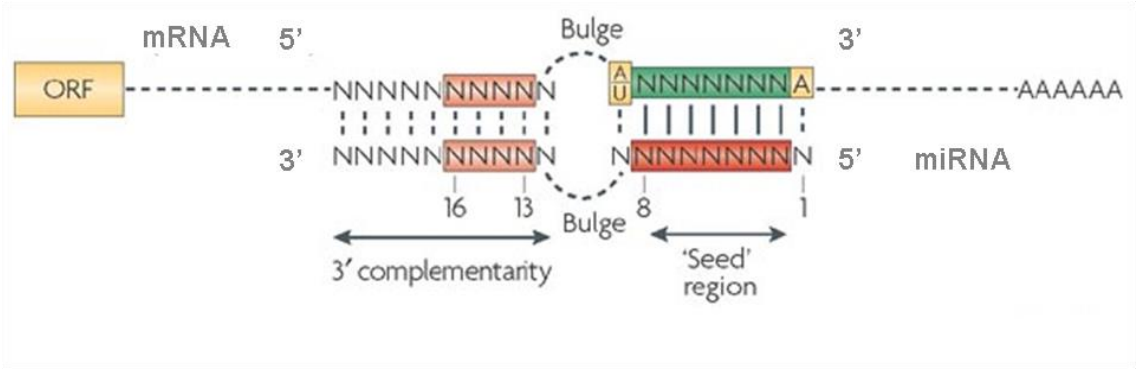

Figure 1.7. Representation of miRNA:mRNA interaction in animals. Binding of miRNAs to target mRNAs requires perfect complementarity between the seed region (nucleotides 2-8, red rectangle) and the 3'UTR of the mRNA (green rectangle). Possible base pairing involving the 3' end of the miRNA might occur (pink rectangles), contributing for a best stabilization of the miRNA:mRNA duplex. The presence of a central bulge prevents the cleavage of the mRNA by Ago2. *In Filipowicz et al. (2008)*.

This limited complementarity between miRNA and its target mRNA was proven to be an advantage in gene expression regulation, since the short length of miRNA seed region allows the simultaneous inhibition of hundreds of target mRNAs (Baek et al., 2008; Lewis et al., 2005; Selbach et al., 2008). Therefore, the biological effects of miRNAs can reflect synergistic effects through simultaneous regulation of different targets. The rules governing miRNA:mRNA Watson–Crick base pairing are quite complex, and though perfect pairing between seed regions and target 3'UTRs is crucial for gene regulation, 3′-end pairing might contribute to target recognition (Fig. 1.7), particularly when sites have weaker miRNA seed matches (Li et al., 2008). Imperfect miRNA:mRNA interactions with central bulges (nucleotides 9–12) facilitate translational

inhibition or exonucleolytic mRNA decay (Fig. 1.7), whereas the outcome of highly complementary binding sites is normally target regulation and slicing (Brodersen and Voinnet, 2009). Furthermore, the presence of multiple binding sites is though to enhance the degree of repression by miRNAs.

In the last few years, our knowledge about miRNA functions has greatly increased and some surprising modes of action have been identified. For instance, Zardo and colleagues demonstrated that miRNAs can regulate gene expression also at the transcriptional level, as was the case for miR-223 binding to the NFIA (nuclear factor I/A) promoter which repressed its transcription during granulopoiesis (Zardo et al., 2012).

Nevertheless, while miRNA-mediated gene regulation mechanisms are still under investigation, several other factors can influence miRNAs effects on their target miRNAs, but are not within the scope of this work and therefore will not be addressed here.

1.1.7. miRNA Functions

As key regulators of gene expression, miRNAs have been implicated in a variety of developmental, physiological and pathological processes, including cell proliferation, apoptosis, differentiation and cell fate decisions (Erson and Petty, 2008; Fatica et al., 2008; Ivey and Srivastava, 2010; Wang et al., 2013b; Wienholds and Plasterk, 2005; Wu et al., 2012; Xiong et al., 2010). Insights about the role of miRNAs in animals have been obtained through diverse approaches, including gain- and loss-of-function genetic screens in *C. elegans* and Drosophila (Brennecke et al., 2003; Lee et al., 1993; Reinhart et al., 2000; Slack et al., 2000), reverse genetic approaches by miRNA knockout or knockdown (Lee et al., 2005; Meister et al., 2004), miRNAs expression profiling (Bak et al., 2008; Chen et al., 2005a), mRNA target identification and validation (Jia et al., 2011; Wang et al., 2013a) and bioinformatics inference (Liu et al., 2012). Moreover, crucial evidences about miRNA essential roles resulted from approaches involving silencing of Dicer and consequent mature miRNAs depletion in several model systems. While Dicer-mutant mouse embryos failed to produce multipotent stem cells and ~50% died by day 7.5 (Bernstein et al., 2003), inactivation of zebrafish Dicer resulted in an inhibition of pre-miRNA

processing, loss of miRNA accumulation and abnormal morphogenesis, which was mainly attributed to miR-430 loss of function (Giraldez et al., 2005; Wienholds et al., 2003). Both studies evidenced the importance of miRNAs in vertebrate development. In particular organs and systems, conditional deletion of Dicer enabled to understand the role of miRNAs during embryonic stem cell proliferation (Murchison et al., 2005), formation of normal cartilage and bone (Gaur et al., 2010; Kobayashi et al., 2008), heart function (Roy et al., 2013) and neuronal function (Dorval et al., 2012).

Several studies have also implicated miRNAs in disease (Carissimi et al., 2009; Saito and Saito, 2012) and, in this field, cancer has been in the spotlight in the last years. In such pathological contexts, miRNAs can behave either as tumour suppressors or oncomiRs that become deregulated. Also, altered expression levels of *Drosha*, *DGCR8*, *Dicer*, *XPO5*, *Ago2* and *TRBP*, which are crucial genes for the miRNA biogenesis machinery (see section 1.1.5), have been correlated with several cancer types including ovarian, lung, breast and prostate cancer (reviewed in Huang et al., 2014). This strongly suggests that the majority of miRNAs is implicated in cancer, supporting numerous studies that already demonstrated the tumorigenic effects of particular miRNAs (reviewed in Anwar and Lehmann, 2014; Bi and Chng, 2014; Calin and Croce, 2006; Christodoulatos and Dalamaga, 2014; Esquela-Kerscher and Slack, 2006; Pedroza-Torres et al., 2014).

Ultimately, miRNAs have emerged as biomarkers for cancer and other pathologies (Cao et al., 2014; Christodoulatos and Dalamaga, 2014), due to their ability to circulate in blood, either in blood cells or in a free state, transported by exosomes, lipoproteins or bound to proteins (Khalyfa and Gozal, 2014). These findings opened new doors for non-invasive miRNA-based detection strategies for disease diagnosis and prognosis, as well as for the development of new therapeutic tools (De Guire et al., 2013).

1.1.8. Investigating miRNA functions

1.1.8.1. miRNA Profiling

Characterization of miRNA temporal and spatial expression patterns is essential to understand miRNA function. Several studies have demonstrated that high expression of miRNAs in a specific cell type, tissue or developmental stage normally correlates with a regulatory function of that miRNAs in that system. In that sense, a range of techniques is currently available for profiling miRNAs according to their expression levels. Northern blotting was used in the first studies, where small RNAs from different samples were detected through labelled DNA probes complementary to miRNA sequences (Ambros et al., 2003; Lagos-Quintana et al., 2001). The use of highly sensitive and specific Locked Nucleic Acid (LNA) modified probes has improved this technique (Kloosterman et al., 2006; Válóczi et al., 2004) and further enabled to localize / detect miRNAs in tissues by *in situ* hybridization (ISH) (Kloosterman et al., 2006). None of these techniques however, allowed the detection of low abundant miRNAs and in fact, both require hard labouring when characterizing several miRNAs.

Recently, development of easy and high-throughput quantification methods has facilitated large-scale expression profiling of miRNAs. In this regard, microarray analysis was shown to be powerful method that was based on antisense oligonucleotides specifically binding to previously labelled mature miRNAs. This originated a signal which intensity can be quantified from scanned images using appropriate software (Baskerville and Bartel, 2005). This technique is still currently employed for miRNA profiling in different cell types, tissues, developmental stages and also diseases. Another currently used technique is the real time-PCR (qPCR), a highly sensitive method that allows the quantification of mature miRNAs with higher accuracy. Although this technique is widely used to validate microarray data, its major disadvantage concerns its inability to quantify miRNAs (and transcripts in general) in a high-throughput manner

The most recent advance in miRNA profiling is next-generation sequencing, a technique that allows quantification and sequencing of up to

million molecules. Its accuracy and sensitivity allows the detection of very low abundant miRNAs. The major drawback of this technique is the cost of each analysis. Even though, there are numerous examples of the successful use of this technique in miRNA profiling in different model organisms as reviewed by Cullum et al. (2011).

1.1.8.2. miRNA Targets: Identification and Validation

Functional characterization of miRNAs is largely based on the identification of their target genes. This can be a true challenge since a single miRNA can regulate hundreds of targets and one gene is normally modulated by more then one miRNA (Bartel, 2004; Filipowicz et al., 2008; Pillai, 2005; Rajewsky, 2006). Furthermore, it is now thought that most genes are regulated / controlled by miRNAs (Friedman et al., 2009). The majority of animal miRNAs pair imperfectly with their cognate targets and the identification of important biological targets is complex. The key element for identification of miRNA:mRNA interactions relies on the perfect pairing of the seed region (nucleotides 2-8) of the miRNA to the 3'UTR of the target mRNA. In the last years, several bioinformatics algorithms based on seed pairing and evolutionary conservation have been developed and became a powerful tool to identify miRNA targets (Bartel, 2009; Friedman et al., 2009; Lewis et al., 2003b, 2005). Among these are TargetScan, PicTar, miRanda, RNAhybrid, and many others. These algorithms use at least one of the following criteria to predict mRNA targets: (i) perfect or near perfect complementarity to the miRNA seed region; (ii) evolutionary conservation of the binding site (BS); (iii) free energy of the miRNA:mRNA duplex; (iv) multiple BSs in one single mRNA; (v) mRNA sequence features outside the target site (Chen and Rajewsky, 2006; Duursma et al., 2008; John et al., 2004; Lewis et al., 2003b, 2005; Thomas et al., 2010; Zhao et al., 2005). Algorithms such as TargetScan and PicTar, initially relied on the seed region in miRNA targeting. Thus, for example, TargetScan requires a perfect match to at least 7 nt of the seed sequence and evolutionary conservation is also considered (Friedman et al., 2009; Lewis et al., 2003b, 2005). In addition, a 'context score' is provided, based on features in the surrounding mRNA; targets with a high context score or multiple predicted BSs

are more prone to be truly regulated by a given miRNA (Grimson et al., 2007). Recently, this algorithm extended target prediction to zebrafish (TargetScanFish) allowing a deeper analysis on evolutional perspective (Garcia, et al. 2011; Grimson et al., 2007; Lewis et al., 2005; Ulitsky et al., 2012). Other algorithms, such as PITA, account for target site accessibility (Kertesz et al., 2007), estimating the free energy cost to unfold secondary structures surrounding the mRNA target site. An important advantage of PITA is the option for uploading both miRNAs and mRNAs of interest, allowing the study of new non-annotated miRNAs and genes unavailable in databases.

Robust comparisons of prediction algorithms are missing and, although many experimentally validated targets were found to be enriched in exact miRNA seed matches, high-throughput experimental analyses of Ago-bound miRNA-mRNA pairings ('pull-down') suggest that around 25%–45% of BSs lack a perfect seed match (Chi et al., 2009), indicating that filtering target genes by perfect seed mach criteria most likely eliminates *bona fide* targets. Regardless of some limitations, prediction algorithms are crucial starting points for the identification of putative miRNA targets. Nevertheless, posterior experimental validation using an *in vitro* and/or *in vivo* system has to be addressed in order to identify true miRNA targets. Experimental validation of targets is labour intensive and is normally based on: (i) validation of miRNA:miRNA interactions through reporter assays; (ii) confirmation of miRNA and target mRNA co-expression; (iii) miRNA effect on target protein and (iv) miRNA effects on target biological function (Kuhn et al., 2008). Outcome of prediction algorithms may result in multiple putative miRNA BSs, which can be tested using reporter assays. Briefly, the 3'UTR of the target gene is inserted in a plasmid downstream of the luciferase (Firefly or Renilla), green fluorescent protein (GFP) or another reporter open reading frame (ORF). Reporter construct and a mimic of the miRNA of interest are then transiently transfected into a host cell and the activity of the reported is measured. Alternatively, the reporter construct can be transfected into cells endogenously expressing the relevant miRNA. Binding of the miRNA to its target will repress reporter protein production, decreasing reporter activity, which is then normalized and compared to several controls (Kuhn et al., 2008). Further confirmation of *bona fide* BSs can be performed through point mutation approach or by specific knockdown of

miRNAs of interest. In order for the miRNA to repress its target, they both have to be co-expressed in the same cell / tissue which is normally demonstrated by Northen blotting, qPCR or *in situ* hybridization (Inose et al., 2009; Wang et al., 2013a). If a particular mRNA is a real target of a specific miRNA, modulation of miRNA levels should result in alteration of target protein levels. Therefore, a typical approach to validate biological targets is gain- or loss-of-function experiments followed by Western blot using a specific antibody against protein of interest (Wang et al., 2013a). Gain-of-function experiments consist in transfecting cells or microinjecting embryos with a miRNA mimic (oligonucleotide with an identical sequence to the mature or pre-miRNA). In loss-of-function experiments, a miRNA of interest is knocked down by delivering antisense oligonucleotides (antagomiRs, anti-miRs or morpholinos (MO)), which can block miRNA processing of the pri-miRNA or the pre-miRNA and/or impair the activity of mature miRNAs (Kloosterman et al., 2007; Velu and Grimes, 2012). Furthermore, generation of transgenic *in vivo* models has proved to be a valuable tool to study the function of miRNAs (Giraldez et al., 2005; Wang et al., 2013a; Watanabe et al., 2008).

It is commonly accepted that when a miRNA is up-regulated its targets will be down-regulated; similarly, miRNA knockdown will result in the up-regulation of its targets. Since miRNAs regulate gene expression by both translational repression and mRNA cleavage (Eulalio et al., 2008; Schmitter et al., 2006), the effect of a miRNA should be assayed at both protein and mRNA level. Once miRNA regulation of a target gene has been experimentally confirmed, a change in a specific biological function should be investigated. This can be challenging because in many systems miRNAs actions are redundant and their functions are assumed by other miRNAs of the same family.

In summary, the number of validated targets has increased in the past years but is still very low considering the number of expected targets predicted by bioinformatics tools. It is noteworthy however, that in the last years these approaches have been successfully applied to identify disease-specific miRNAs (Carissimi et al., 2009; Hong et al., 2012; Ventura and Jacks, 2009) allowing to uncover novel therapeutic strategies. In that sense, the search and identification

of new miRNAs, and validation of their target genes, is an important and critically imperative field of research.

1.2. Skeletal Development and Maintenance

1.2.1. Overview

The vertebrate skeleton is composed of multiple elements of various shapes and origins spread throughout the body. Skeletogenesis, the process of skeleton formation during vertebrate development, starts when mesenchymal cells from ectoderm and mesoderm migrate to particular positions in the body and commit to skeletal fate. The skeleton of vertebrates is composed by two distinct tissues, cartilage and bone, and three main cell types, chondrocytes (associated to cartilage), osteoblasts and osteoclasts (both found in bone) (Fig. 1.8). While osteoclasts derive from the hematopoietic lineage, chondrocytes and osteoblasts derive from multipotent mesenchymal cells, thus sharing a common progenitor (reviewed in Karsenty & Wagner, 2002). Both chondrocytes and osteoblasts participate in a process called endochondral bone formation that occur mainly in long bones of vertebrates and where bone is initially formed from a cartilage template. Conversely, flat bones are formed by another process, intramembranous ossification, where osteoblasts differentiate directly from condensed mesenchymal precursors (Crombrugghe et al., 2001; Karsenty and Wagner, 2002). Several factors elaborately control the process of osteochondroprogenitors proliferation and differentiation that ultimately results in cartilage and/or bone formation. A resumed description of these molecules is presented in the next sections.

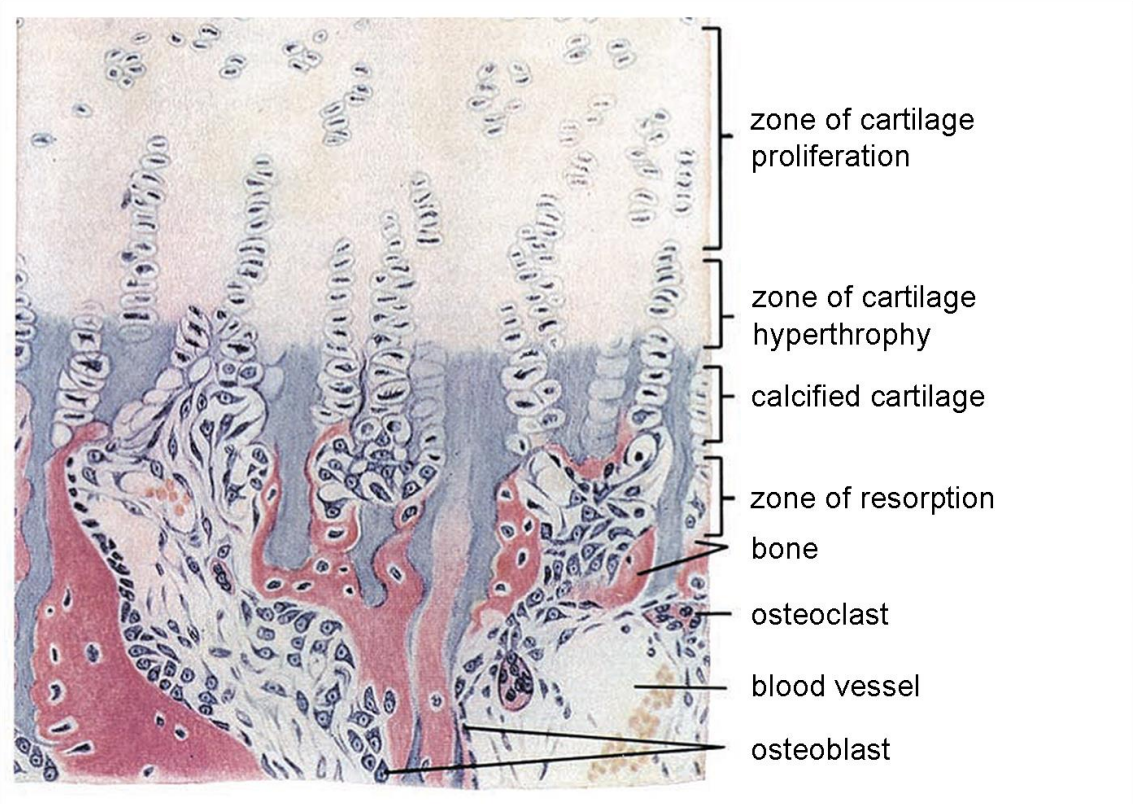

Figure 1.8. Endochondral bone formation and bone remodeling in mammalian systems. Proliferative and hyperthrophyc chondrocytes are observed in the zone of cartilage proliferation and hyperthrophy, respectively. Calcified cartilage is eventually replaced by bone, upon blood vessel invasion and osteoblast recruitment. Bone resorption performed by a multinucleated osteoclast is indicated in the bottom of the image. H&E, x280. *Adapted from Ross and Pawlina (2011).*

1.2.2. Control of Chondrogenesis

Mammalian skeleton is mostly formed through endochondral bone formation, including the vertebral column and long bones from limbs. Chondrogenesis, the process of cartilage formation, is a multi-step cellular process essential for endochondral bone formation. It consists in commitment of undifferentiated mesenchymal cells to the chondrogenic lineage, cellular condensation, chondrocyte proliferation, matrix proteins production, maturation and hypertrophic conversion, followed by bone replacement (Fig. 1.9) (Karsenty and Wagner, 2002; Kronenberg, 2003; Michigami, 2013). Expression of adhesion molecules such as N-cadherin facilitate aggregation (Oberlender and Tuan, 1994) during cellular condensation, a process governed by transforming growth factor-beta (TGF-β), Wnt canonical signalling and sex determining region Y-box (Sox) 9 (Akiyama et al., 2000, 2004; Chun et al., 2008; Tuli et al., 2003). Then,

aggregated cells stop expressing adhesion molecules, restart proliferation, and begin to produce an extracellular matrix (ECM). Proliferating chondrocytes form organized parallel columns in growth zones, and express several collagens, including type II, IX, and XI, integrins and proteoglycans, such as aggrecan (Michigami, 2014). Early chondrocyte differentiation is driven by Sox5, Sox6, and Sox9 transcription factors (Akiyama, 2008; Goldring et al., 2006). Expression of Sox9 begins at the mesenchymal osteochondroprogenitors stage, preceding expression of Sox5 and Sox6. The three TFs cooperate for the activation of genes specific of proliferating chondrocytes, such as collagen type II alpha 1 (Col2a1) (Lefebvre et al., 1997). Bone morphogenetic protein (BMP), Indian hedgehog (Ihh) and fibroblactic growth factor (FGF) are likely to cooperate for the activation of Sox9 in precursor and/or primary chondrocytes (Murakami et al., 2000; Zeng et al., 2005a), whereas Wnt/β-catenin signalling was found to block Sox9 and thus low levels of β-catenin seems to be required for chondrocyte lineage commitment (Akiyama et al., 2004; Hill et al., 2005). When chondrocytes become hypertrophic, they begin to produce high levels of alkaline phosphatase and type X collagen. Ultimately, terminally differentiated chondrocytes undergo apoptosis, and the cartilaginous matrix is mineralized and replaced by bone (Kronenberg, 2003; Michigami, 2013). These chondrocytes express vascular endothelial growth factor (VEGF), which induce blood vessel invasion, and matrix metalloproteinases (MMPs), which are important for cartilaginous matrix degradation (Stickens et al., 2004; Zelzer et al., 2004). Hypertrophic maturation of chondrocytes requires the expression of runt-related transcription factor (Runx) 2 and Runx3 transcription factors as well as a decrease of Sox proteins (Yoshida and Komori, 2005; Yoshida et al., 2004). Runx2 is known to drive transactivation of Ihh, collagen type X alpha 1 (Col10a1) and matrix metallopeptidase 13 (collagenase 3) (MMP13) (Selvamurugan et al., 2004; Yoshida et al., 2004; Zheng et al., 2003). Recently, Sox9 was shown to repress Runx2 and β-catenin signalling, thus inhibiting chondrocytes hypertrophy (Dy et al., 2012). The basic helix-loop-helix (bHLH) twist family transcription factor 1 (Twist-1) also functions as a repressor of Runx2 in the perichondrium thus controlling chondrocyte maturation (Bialek et al., 2004).

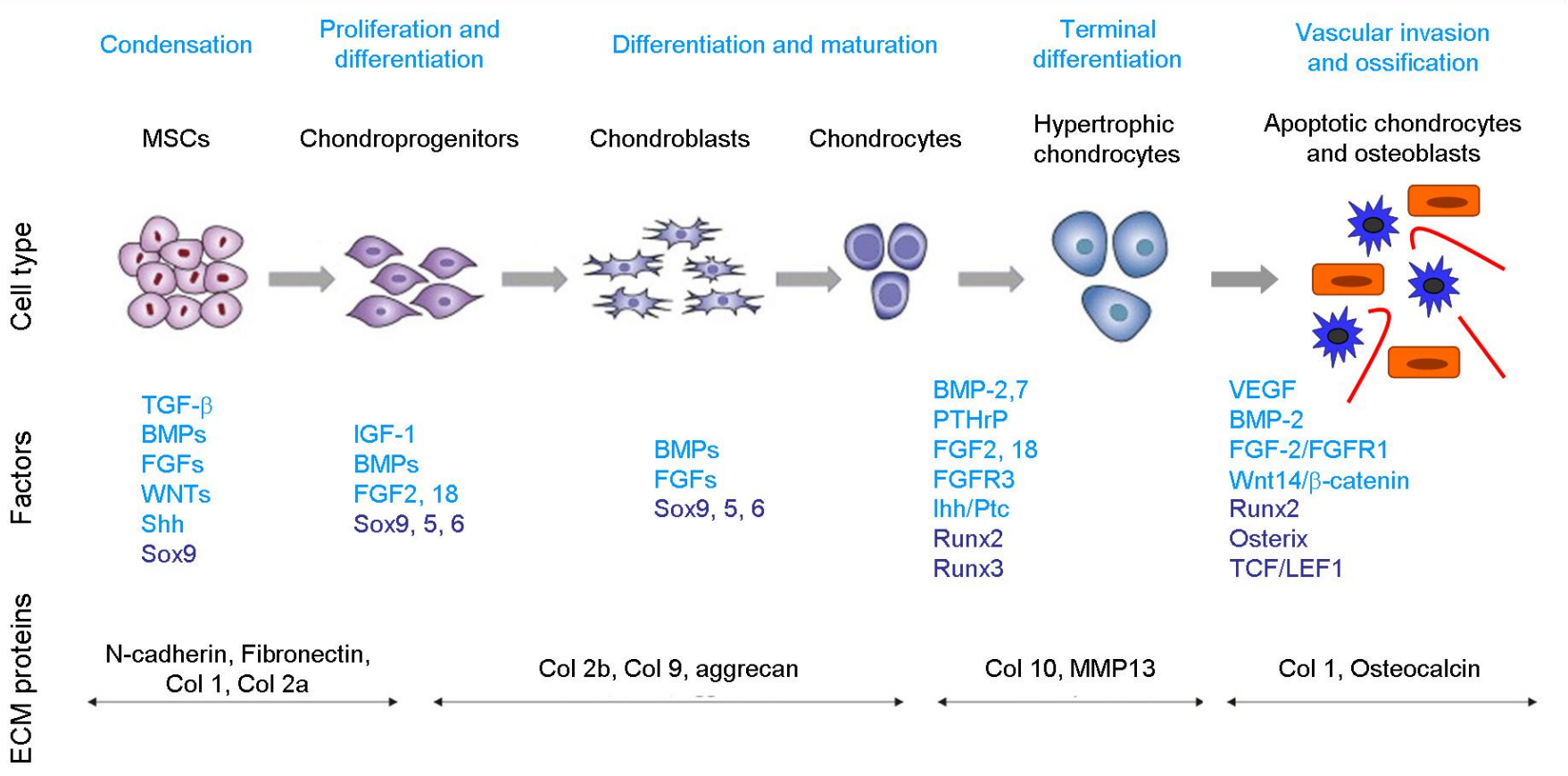

Figure 1.9. Sequence of multi-step events during chondrogenesis. The different stages of chondrogenesis are represented schematically and associated main growth and differentiation factors (in light blue) and the transcription factors (dark blue) at each stage are indicated. Important extracellular matrix proteins which characterize the different stages are shown at the bottom. Adapted from (Kelc et al., 2013).

In general, in the last decades, chondrogenesis was shown to be a highly complex process, tightly regulated by numerous transcription factors, growth factors and signalling pathways. Nevertheless, recent advances in molecular and genetic research have clearly indicated that this complexity is extended to the post-transcriptional level. Such regulatory mechanisms in chondrogenesis are described next.

1.2.2.1. Post-transcriptional Control of Chondrogenesis

The crucial roles of miRNAs in chondrogenesis were first revealed when severe skeletal growth defects were observed in transgenic mice lacking *Dicer* gene in cartilage (directed by Col2a1 promoter) (Kobayashi et al., 2008). Cartilage growth plates from this *Dicer-null* mice presented a general decreased in chondrocyte proliferation, while differentiation was accelerated (Kobayashi et al., 2008). Since then, an increasing number of specific miRNAs was shown to regulate chondrocyte differentiation. The cartilage-specific miR-140 was shown to positively regulate chondrogenesis in zebrafish through platelet-derived growth factor (PDGF) signalling (Eberhart et al., 2008). In mice, miR-140 deletion not only resulted in decreased statures, but also induced osteoarthritis-like phenotypes either related to age or stress induced in surgical models. The main target associated to these changes was a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif 5 (Adamts-5). With these results, miR-140 was associated to regulation of both development and homeostasis of cartilage (Miyaki et al., 2010). Another important example of miRNAs involvement in chondrogenesis was demonstrated in human chondrocytes from healthy donors. Apparently, in this system, miR-675 was up-regulated by Sox9 during chondrogenesis, suggesting a crucial involvement in this process. This miRNA up-regulated COL2a1 expression through a mechanism yet to be determined (most likely via targeting a col2a1 repressor) (Dudek et al., 2010). In two other studies developed *in vitro*, two negative regulators of chondrogenesis were identified: miR-199a, by targeting Smad1 (Lin et al., 2009), and miR-145, which inhibits Sox9 (Yang et al., 2011a). Nevertheless, information regarding miRNA involvement in chondrogenesis is still scarce. In that sense, miRNA arrays-based studies are

currently being developed which is expected to bring new insights into the complex regulatory network controlling chondrogenesis.

1.2.3. Control of Osteogenesis

As previously described, most bones in vertebrate skeleton are originated from endochondral ossification, while only a few, including the skull, are formed by intramembranous ossification. During this process, osteoblasts derive from mesenchymal stem cells (MSCs), which can also differentiate into chondrocytes, adipocytes or myocytes, depending on specific signalling pathways activation and/or inhibition (de Gorter and ten Dijke, 2013). Commitment of MSCs into osteochondroprogenitors seems to require Sox9 expression since its inactivation before mesenchymal condensation results in complete absence of both cartilage and bone and abolishes Runx2 expression (Akiyama et al., 2002). Since they share a common precursor, chondrocytes and osteoblasts also share several regulatory factors and signalling pathways during skeletogenesis including BMPs, transforming growth factor beta (TGF-β), Wnt, Hedgehog (Hh), parathyroid hormone (PTH), insulin-like growth factors (IGFs) and FGFs.

The commitment of osteochondroprogenitors to the osteoblast lineage is driven by Runx2, the master regulator of osteogenesis acting in all stages of osteoblast differentiation (Fig. 1.10) (Komori et al., 1997; Otto et al., 1997). The role of Runx2 in osteogenesis was first evidenced by Runx2-deficient mice which skeleton was fully deprived of osteoblasts (Komori et al., 1997) and studies of cleidocranial dysplasia in which heterozygous loss, insertion, deletion or mutations of the Runx2 gene was shown to result in defective bone formation (Lee et al., 1997; Mundlos et al., 1997). Exactly how many key factors are involved in the process of MSCs commitment into the osteoblast lineage is yet to be determined. However, this process seems to involve signals from BMP, Wnt, FGF and Hedgehog pathways, which are thought to participate in the control of Runx2 expression (Karsenty et al., 2009; Komori, 2011). For instance, the absence of Ihh in MSCs results in down-regulation of Runx2, which consequently inhibits commitment into osteoprogenitors (St-jacques et al., 1999). Also, canonical Wnt signalling up-regulation by glycogen synthase

kinase-3 beta (GSK3β) knockdown in mice induces Runx2 expression, and as a consequence, bone formation increases (Kugimiya et al., 2007). Furthermore, expression of β-catenin in MSCs was demonstrated to favour osteochondroprogenitor cell differentiation into osteoblasts and to prevent their differentiation into chondrocytes (Day et al., 2005; Hill et al., 2005).

Following lineage commitment, osteoprogenitor cells undergo proliferation and differentiate into pre-osteoblasts, a process governed by Runx2 and characterized by the production of proteins such as collagen type I, fibronectin, histones, proto-oncogene c-Fos (c-Fos) and c-Jun (c-Jun) and cyclin-dependent kinase inhibitor 1 (p21) (Shalhoub et al., 1989). At this stage, BMPs play a significant role, increasing alkaline phosphatase (ALP) activity and osteocalcin (Oc) synthesis (Neve et al., 2011). After growth arrest, pre-osteoblast differentiation proceeds towards osteoblast maturation, a process in which the zinc finger transcription factor Osterix (Osx, also known as Sp7) plays a critical role (Fig. 1.10). Like Runx2, Osx is required for osteoblast differentiation and is essential for bone formation (Nakashima et al., 2002). Osx-null mice die at birth due to general lack of mineralized structures. Remarkably, despite its importance in bone formation, information regarding its transcriptional regulation, functional partners or targets is limited. Nevertheless, Osx is known to act downstream of Runx2 (Nakashima et al., 2002; Nishio et al., 2006), to interact with nuclear factor of activated T-cells cytoplasmic calcineurin-dependent 1 (NFATc1) to activate collagen type I alpha 1 (*Col1a1)* (Koga et al., 2005) and to repress the canonical Wnt signalling (Zhang et al., 2008) during osteoblast differentiation. Inhibition of Wnt/β-catenin signalling, which plays a crucial role in stimulating osteoblast proliferation, by SP7, might explain its negative effect on osteoblast proliferation, which favours differentiation (Zhang et al., 2008). Early osteoblast maturation is marked by the expression of genes such as ALP, bone sialoprotein (BSP) and type I collagen. At this stage, osteoblasts begin to produce an organic non-mineralized matrix called osteoid. At the end of differentiation, osteoblasts express genes implicated in mineralization of the ECM such as Oc, osteopontin (OPN) and collagenase..

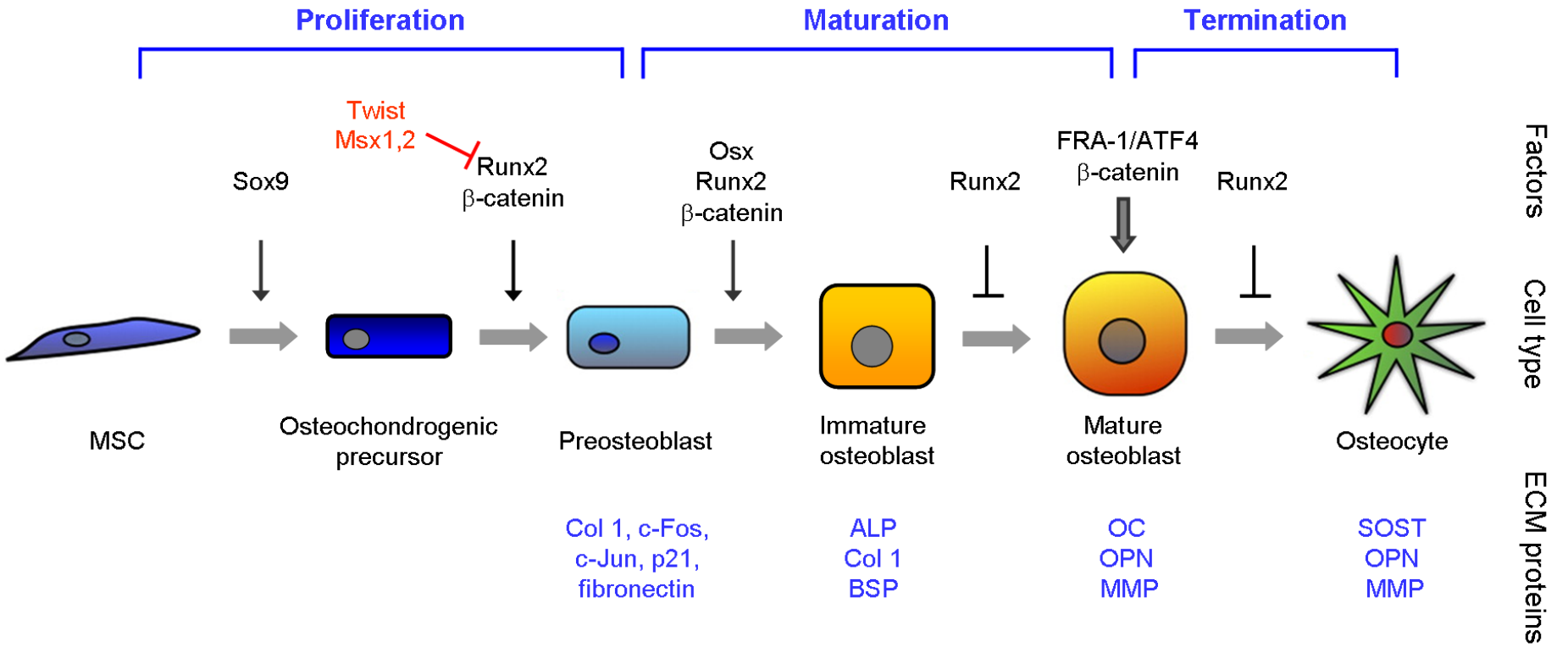

Figure 1.10. Sequence of multi-step events during osteogenesis. The different stages of osteogenesis are represented schematically and associated main effectors at each stage. Important extracellular matrix proteins which characterize the different stages are shown in blue at the bottom. Lines with arrowheads indicate a positive action and lines with bars indicate an inhibition. Vertical arrow indicates stimulation of bone matrix genes expression.

A major regulator of mature osteoblasts is activating transcription factor 4 (ATF4) (Fig. 1.10), a member of the cAMP response element-binding protein (CREB) family of basic leucine zipper domain (B ZIP) proteins. Although dispensable for early osteoblast differentiation, ATF4 is required for osteoblast terminal differentiation and function (Yang et al., 2004). In fact, ATF4 regulates osteoblast ability to synthesize collagen type I (Col 1), the main constituent of the bone matrix, and drives the expression of Oc, by cooperative interaction with Runx2 and special AT-rich sequence-binding protein 2 (SATB2) (Dobreva et al., 2006; Xiao et al., 2005; Yang et al., 2004), and SP7, through a PTH-dependent mechanism (Yu et al., 2009). At the final stage of osteogenesis, matrix mineralization takes place and some osteoblasts become trapped in lacunae within the matrix. These cells, known as osteocytes, are connected by a system of canaliculi and express several inhibitors of the Wnt pathway, including sclerostin (SOST) (Burgers and Williams, 2013). The remaining osteoblasts lie on the surface of bone, constituting bone lining cells (Ehrlich and Lanyon, 2002).

Several other factors and signalling pathways govern osteoblast differentiation, generally by controlling one of the three main osteoblast specific transcription factors. For instance, Twist1 and Twist2 interact with Runx2, inhibiting its binding to DNA and preventing early osteoblast differentiation (Bialek et al., 2004). BMPs are known inducers of osteoblast differentiation and in this regard, BMP-2 was demonstrated to induce the expression of both Runx2 and SP7, and also ALP, Col 1 and Oc (reviewed in Yamaguchi et al., 2008). FGF and Wnt signalling pathways are also implicated in several stages of osteoblast differentiation. Fibroblast growth factor receptor (FGFR) 1 promotes osteoblast early differentiation whereas FGFR2 and FGFR3 support osteoblast maturation. While FGF2, 4 and 8 are essential in skeletogenic mesenchyme, FGF18 promotes osteoblast proliferation and maturation (Ornitz, 2005). The Wnt/β-catenin pathway, not only participates in the osteochondroprogenitor cell fate decision into either osteoblasts or chondrocytes, but also is known to boost osteoblast differentiation (Hill et al., 2005; Karsenty et al., 2009). Conversely, Notch signalling inhibits osteoblastogenesis through several mechanisms,

including inhibition of Runx2 function and Wnt/β-catenin signalling pathway (Zanotti and Canalis, 2010; Zanotti et al., 2008).

In addition to other factors and pathways which were not addressed here (including IGF, TGFβ and parathyroid hormone-related protein (PTHrP) signalling and, CREB, c-*myc*proto-oncogene (c-Myc), activator protein 1 (AP1), e T cell factor/lymphoid enhancer factor (TCF/LEF), specificity protein 1 (SP1), c-Fos, Jun and signal transducer and activator of transcription (STAT) families of transcription factors) several studies have revealed cross-talks between different signalling pathways further increasing the complexity of the mechanisms involved and providing further insight towards a better understanding of osteoblast differentiation (Guo and Wang, 2009; Hartmann, 2006; Jensen et al., 2010; Karsenty et al., 2009; Yamaguchi et al., 2008). Interestingly, this complexity was taken into another level with the discovery of novel players in osteoblastogenesis: the miRNAs.

1.2.3.1. Post-transcriptional Control of Osteogenesis

Like in chondrogenesis, the fact that miRNAs are involved in the control of osteogenesis was first demonstrated following a conditional deletion of Dicer in osteoprogenitors (directed by Col1a1 promoter) and mature osteoblasts (directed by Oc promoter) of mice (Gaur et al., 2010). Ablation of Dicer in osteoprogenitors compromised fetal survival after E14.5, and e*x vivo* studies revealed a general decrease of osteoblast markers and ECM mineralization in committed osteoblasts. Conversely, *in vivo* ablation of Dicer in mature osteoblasts produced viable mice with delayed bone mineralization in early development. Surprisingly, adult mice showed a dramatic increase in bone mass (Gaur et al., 2010). This study suggested different roles for Dicer and miRNAs during distinct stages of skeleton development and osteoblast differentiation. While a diverse set of miRNAs in the fetal skeleton seems to be required for osteogenesis; miRNAs in mature osteoblasts apparently play major roles in regulating bone homeostasis by controlling of bone matrix protein levels in the adult skeleton (Gaur et al., 2010).

In sum, Dicer knockout in mice osteoblasts was very important to demonstrate the general roles of miRNAs in osteogenesis. However, it failed to

establish the functions of particular miRNAs in this process. In recent years, several studies have identified a panel of miRNAs that act either as negative or positive regulators of osteoblastogenesis and bone formation. For instance, miR-206 targeting of connexin43 (Cx43) was shown to inhibit osteoblast differentiation both *in vitro* and *in vivo* (Inose et al., 2009). A set of 11 miRNAs was found to target the master regulator of osteogenesis, Runx2, in both osteoblasts and chondrocytes, and to inhibit osteoblast differentiation (Zhang et al., 2011c). Repression of SP7 by miR-637, was shown to promote commitment of human MSCs into the adipocyte lineage, while it inhibited osteoblast differentiation (Zhang et al., 2011a). Furthermore, miR-214 was shown to repress ATF4, which consequently inhibited osteoblast activity and matrix mineralization *in vitro* and bone formation *in vivo* (Wang et al., 2013a). Regarding positive regulation of osteoblast differentiation, miR-2861 overexpression was shown to enhance osteoblastogenesis in mouse bone marrow stromal cells, while its silencing *in vivo* decreased Runx2 protein levels and inhibited bone formation, by targeting histone deacetylase 5 (HDAC5) (Li et al., 2009a). In another work, Zhang and co-workers demonstrated that miR-335-5p is able to repress dickkopf WNT signalling pathway inhibitor 1 **(**DKK1) and consequently potentiate the Wnt signalling and promote osteogenic differentiation (Zhang et al., 2011b). The miR-29 family was also shown to promote osteoblast differentiation by targeting several inhibitors of osteoblast differentiation (Li et al., 2009b; Trompeter et al., 2013), by regulating several proteins crucial for ECM maintenance (Kapinas et al., 2009; Li et al., 2009b; Rossi et al., 2013) and by potentiating the Wnt canonical signalling through a feedback-loop (Kapinas et al., 2010).

1.2.4. Control of Bone Remodelling

Bone remodelling is a dynamic and continuous process in which bone resorption is coupled to bone formation, through delicate balancing between the number and activities of osteoblasts and osteoclasts. While osteoblasts are responsible for bone formation (see section 1.2.3.), osteoclasts are multinucleated bone-resorbing cells of hematopoietic origin that also undergo distinct stages of differentiation (Fig. 1.11). Briefly, osteoclast progenitors

differentiate into mononuclear tartrate-resistant acid phosphatase (TRAP)-positive cells which then become cathepsin K-positive osteoclasts and differentiate into mature multinucleate osteoclasts (Lerner, 2000). In detail, mononuclear precursor cells are stimulated by c-fms/MCSF (macrophage colony stimulating factor) to express receptor activator of nuclear factor κ B (RANK) (Crockett et al., 2011a); simultaneously, RANK ligand (RANKL) is expressed by osteoblasts in close vicinity with these precursors, in response to PTH and 1,25 Vit D_3 stimulation (Leibbrandt and Penninger, 2008). This results in activation of several transcription factors, e.g. nuclear factor kB (NF-kB), AP1 and NFATc1 (Humphrey et al., 2005), that drive the expression of crucial osteoclast genes, such as dendritic cell-specific transmembrane protein (DC-STAMP), TRAP, cathepsin K, matrix metalloproteinase 9 (MMP-9) and β3 integrin. This process leads to final differentiation and fusion of precursors, which become fully functional multinucleated osteoclasts (Crockett et al., 2011b).

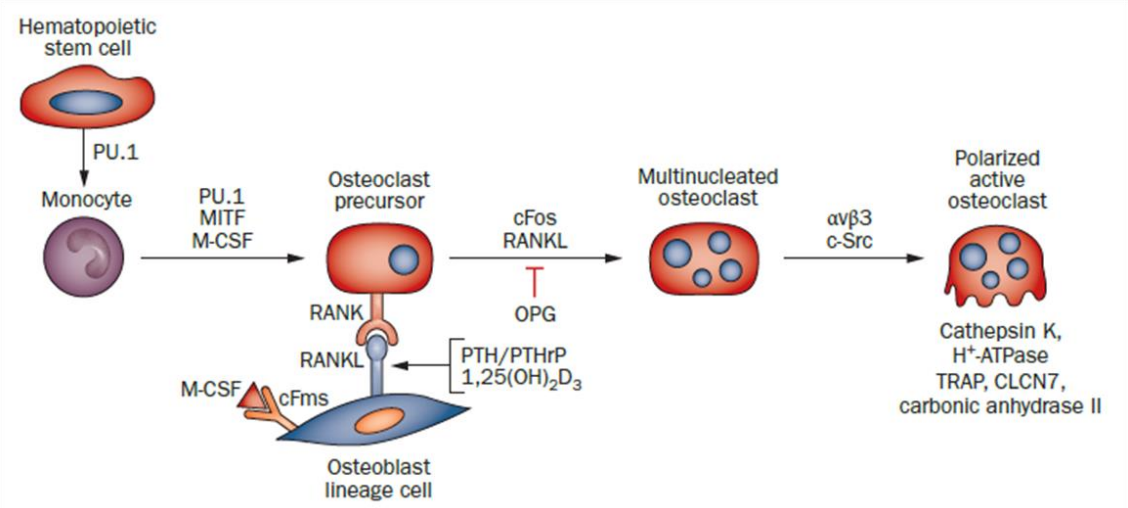

Figure 1.11. Sequence of multi-step events during osteoclast differentiation. The different stages in of osteoclast differentiation from hematopoietic precursor towards active osteoclast are illustrated associated with key transcription and regulatory factors at each stage. *In Lian et al., 2012*.

Similarly to other cells of hematopoietic origin, osteoclasts are recruited to bone surface upon release of cytokines at particular sites where remodelling is necessary / occurring. After adherence, osteoclasts create an acidic

microenvironment in the space beneath them to dissolve the mineralized constituent of the bone matrix. Then, the organic matrix is depredated by cathepsin K (Ross, 2008). In the reversal phase, mononuclear cells assemble the bone surface for new osteoblasts and secrete signals for their recruitment; proliferation and differentiation of new osteoblast will lead to new matrix deposition.

Again, as previously described for chondrocytes and osteoblasts, differentiation of osteoclasts was shown to be governed at the post-transcriptional level by miRNAs. Such regulation is described below.

1.2.4.1. Post-transcriptional Control of Bone Remodelling

The relevance of miRNAs in osteoclast differentiation and function has been confirmed by deletion of Dicer and DGCR8 in mice models, although this was only demonstrated for few miRNAs. Indeed, mice with osteoclast-specific Dicer gene deficiency (directed by cathepsin K) were shown to present a decreased number of active osteoclasts and increased bone mass, suggesting a crucial function of Dicer or Dicer dependent pathway in osteoclasts formation (Mizoguchi et al., 2010). Moreover, mice with osteoclast-specific deletion of DGCR8, the partner in Drosha processing, also had impaired formation of osteoclasts and bone resorption, confirming the previous report (Sugatani et al., 2014). In other studies, four miRNAs with particular roles in the regulation of osteoclastogenesis were identified. Thus, in mouse RAW264.7 cells both knockdown and overexpression of miR-223 inhibited the formation of osteoclast-like cells as induced by RANKL, suggesting that proper levels of miR-223 are required for normal osteoclastogenesis (Sugatani and Hruska, 2007, 2009). Furthermore, while stimulation of osteoclast precursors by macrophage colony stimulating factor (M-SCF) induces proviral integration oncogene (PU.1), which subsequently stimulates both miR-223 and RANK (Fukao et al., 2007), miR-223 was suggested to repress nuclear factor 1 A-type (NFI-A), a suppressor of osteoclastogenesis (Sugatani and Hruska, 2009). Interestingly, miR-21 was also shown to be up-regulated during RANKL-induced osteoclastogenesis, a process mediated by c-Fos. As consequence, miR-21 apparently represses programmed cell death 4 (PDCD4) to establish a positive

feedback loop involving c-Fos/miR-21/PDCD4 to positively regulate osteoclastogenesis (Sugatani et al., 2011). In other studies, miRNAs were shown to be repressors of osteoclastogenesis. For instance, miR-155 is apparently decisive for determining the cell fate of monocytes, promoting macrophage formation in spite of esteoclasts (Blüml et al., 2011). In miR-155 knockout mice, levels of local bone loss were considerably reduced, hich was apparently correlated with reduced number of osteoclasts (Blüml et al., 2011). In another study, miR-146a, a miRNA mostly known as a negative regulator of immune/inflammatory responses, was found to significantly diminish the number of TRAP-positive multinucleated cells in human peripheral blood mononuclear cells, suggesting an inhibition of osteoclastogenesis (Nakasa et al., 2011).

In general, these studies prove that miRNAs are indeed important factors for bone remodelling. However, it also became patent that many others miRNAs remain to be identified. Remarkably, miRNAs have been shown to be able to circulate in blood and promote their effects in an endocrine/autocrine/paracrine manner (De Guire et al., 2013). This feature not only increases the complexity of miRNAs, but also increases their potential to regulate all physiological processes, including those associated to the skeleton. It is widely accepted now that miRNAs can be used as biomarkers for many diseases, including bone-related diseases such as osteoporosis and osteosarcoma (Cao et al., 2014; Yuan et al., 2012).

1.3. Zebrafish as a model

Teleost fish represent one of the most successful groups in vertebrate evolution, with more then 25000 species (Volff, 2005; Witten and Huysseune, 2009a). They are also considered to be good models for the investigation of vertebrate development, including skeletogenesis (Spoorendonk et al., 2010). In that sense, teleosts not only present several anatomic, physiologic and genetic similarities with mammals, allowing extrapolation of information, but also present several experimental advantages, including larvae transparency (crucial for developmental characterization of systems), large progeny and easy manipulation (e.g. transgenic preparation)

Among teleosts, zebrafish (*Danio rerio*) is currently the most used model for studies of vertebrate development, gene and function and human disease (Haffter et al., 1996; Lieschke and Currie, 2007). Initially, zebrafish was used to study embryogenesis and morphogenesis. The research and understanding of these processes led to the implementation of large-scale mutagenic screens which resulted in several disorders, related to zebrafish mutants, and allowing for an extended knowledge of gene function (Dodd et al., 2000). As a research model, zebrafish not only presents the same advantages as other teleosts, including rapid and external embryonic development (Dodd et al., 2000), but it was also one of the first vertebrates to have a sequenced genome. Interestingly, comparison between human and zebrafish genomes revealed that most genes have corresponding orthologs in both species, and share similar chromosomal structures (Howe et al., 2013). This fact, combined with availability of zebrafish transgenic strains and an increasing number of specific molecular tools such as antibodies and dedicated vectors associated to the recent development of several cell lines (Sing-Yee et al., 2014; Vijayakumar et al., 2013), opened new doors for molecular studies using this organism as a model for biological and biomedical research (Dodd et al., 2000; Lieschke and Currie, 2007; Nasevicius and Ekker, 2000; Shin and Fishman, 2002). In that sense, using zebrafish to investigate miRNAs was one of the most recent examples of the suitability of this model in biomedical and biological research (Fjose and Zhao, 2010). Such studies are briefly described in the following section.

1.3.1. Zebrafish and miRNAs

Since the beginning of miRNA research, it was demonstrated that most miRNAs are conserved across vertebrate evolution (Guerra-Assunção and Enright, 2012). This fact not only suggested that miRNA functions should be conserved in vertebrates, but also indicated that alternative vertebrate models, including zebrafish, could be used to facilitate the investigation of miRNAs. In fact, the first models that were used to elucidate the general roles of miRNAs were zebrafish specimens lacking Dicer. Apparently, targeted selected gene inactivation of Dicer 1 resulted in impaired miRNA expression and developmental arrest around day 10 post-fertilization (dpf) (Wienholds et al., 2003). Although the existence of maternally produced Dicer enzyme still enabled generation of mature miRNAs during the first few days, developmental arrest in subsequent stages was important to evidence the crucial roles of miRNAs in early development. Conversely, maternal–zygotic dicer mutants (MZdicer) were incapable of producing mature miRNAs (Giraldez et al., 2005), causing morphogenesis defects. In this study, several miRNAs were screened for a possible rescue of MZdicer zygotic defects. Interestingly, microinjection of miR-430 duplexes partially rescued this phenoptype, revealing a critical role for this miRNA in morphogenesis. In a subsequent study, it was demonstrated that miR-430 family members bind to several hundred maternal mRNAs leading to their deadenylation and degradation (Schier and Giraldez, 2006). It was concluded that the absence of miR-430 in homozygous MZdicer embryos should delay degradation of maternal mRNAs and should consequently affect early development.

Many other miRNAs were identified in zebrafish, at first by sequencing of small-RNA cDNA libraries (Chen et al., 2005b; Kloosterman et al., 2006) and, more recently, by next-generation sequencing (Soares et al., 2009). Determining miRNAs expressions in zebrafish revealed high tissue specificity, suggesting specific roles in tissue differentiation and identity but also evidenced specific functions in zebrafish development. For instance, miR-196 was shown to be a regulator of axial patterning and pectoral appendage initiation in zebrafish (He et al., 2011). In heart development, miR-218 seems to be a crucial mediator of T-box 5 (Tbx5), and its dysregulation should be responsible

for severe cardiac abnormalities (Chiavacci et al., 2012). In regenerating zebrafish fins, miR-203 was shown to repress Lef1, an inhibitor of the canonical Wnt signalling, consequently blocking this process (Thatcher et al., 2008). These and many more studies evidence that zebrafish is a valuable model for miRNA research.

1.4. Objectives

The main objectives of this work were to identify and characterize relevant miRNAs in bone formation and homeostasis using fish as models and mammalian systems for comparison and validation of results. In this regard, a combined literature search and preliminary experimental data revealed a set of miRNAs with a putative involvement in different stages of bone formation and/or homeostasis. Preliminary experiments included analysis of selected miRNAs in different fish bone systems. This thesis focused on the investigation of three miRNAs that highlighted from the initial set of identified miRNAs.

At the beginning of this work, I integrated a pilot study ongoing in our laboratory at that time, which allowed me to acquire experience in some of the techniques described in this thesis and to develop some expertise in miRNA work. In particular, I was involved in the development of stable cell clones overexpressing miRNAs and in the characterization of miRNA gene expression through real-time quantitative PCR. This study, in which I am co-author, involved the investigation of miR-20a role in osteogenesis, through the characterization of three stable clones overexpressing this miRNA, in a fish bone-derived cell system and is now published (**ANNEX I**).

Regarding the three miRNAs selected for this investigation, first, we focused on miR-223 (**CHAPTER II**), a miRNA that has been associated with bone remodelling in mammalian systems, where it was shown to regulate cell fate decisions of the hematopoietic lineage. In this regard, although miR-223 was shown to be a crucial miRNA for osteoclastogenesis, its role in this process was so far poorly characterized. Therefore, we evaluated i) the suitability of zebrafish for the investigation of miR-223 function in vertebrates, and its particular involvement in ii) hematopoiesis and iii) osteoclastogenesis, always in direct comparison with mammalian systems. For that, miR-223 conservation was investigated regarding its gene organization, primary structure, secondary structure of the precursor, and genomic context. MiR-223 spatial and temporal distribution and predicted targets were also explored.

In a second stage of this work, we studied the biological role of miR-29a (**Chapter III**), a bone-related miRNA that was quite extensively investigated in mammals, but for which no mineralogenic effects where so far demonstrated. Thus, we investigated the biological effects of miR-29a through gain-of-function experiments in a fish bone-derived cell line, the ABSa15 cells, capable of *in vitro* mineralization. For a better understanding of the involved mechanisms and possible conservation of processes from mammals to fish, we investigated the expression of genes associated to osteogenic differentiation. The main targets of miR-29a in mammalian osteogenesis were also explored. Finally, miR-29 conservation in vertebrates was investigated regarding its sequence homology, gene synteny, expression patterns and target conservation.

In the third and final stage of this work, we explored miR-214 putative roles in vertebrate skeleton formation (**Chapter IV**). MiR-214 was previously found to regulate Hedgehog signalling, a crucial pathway in bone formation. Although our initial hypothesis of miR-214 involvement in osteogenesis was recently demonstrated by Wang et al. (2013) we proceeded with our investigation focusing on other skeletal-related processes, including chondrogenesis. We started by studying miR-214 expression throughout zebrafish development and collected evidences suggesting a possible involvement in chondrogenesis. Then, we investigated human and zebrafish miR-214 promoters for the presence of conserved binding sites for transcription factors associated to osteogenesis and chondrogenesis. At last, in order to demonstrate an involvement of miR-214 in chondrogenic differentiation, we performed miR-214 gain-of-function experiments in chondrocytic ATDC5 cells. Several chondrogenesis marker genes and putative targets of miR-214 were explored.

CHAPTER 2

Evidence for conservation of miR-223 in zebrafish (Danio rerio): implications for function

CHAPTER 2 • Evidence for conservation of miR-223 in zebrafish (*Danio rerio*): implications for function

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2.1. Abstract

MicroRNAs (miRNAs), an abundant and conserved class of small RNAs, have been shown to play important regulatory functions by interacting with the 3' untranslated region (UTR) of target mRNAs. Through this mechanism, miR-223 was shown to post-transcriptionally regulate genes involved in mammalian haematopoiesis, both in physiological and pathological contexts. Essential for normal myelopoiesis in mammals, miR-223 promotes granulocyte, osteoclast and megakaryocyte differentiation and suppresses erythropoiesis. There is however a general lack of knowledge regarding miR-223 function in other vertebrates, which could help to clarify its role in other processes, such as development. In this work, we explored the functional conservation of miR-223 using zebrafish as a model. We show that mir-223 gene structure and genomic context have been maintained between human and zebrafish. In addition, we identified 22 novel sequences of miR-223 precursor and demonstrate that it contains highly conserved domains among vertebrates, suggesting function preservation throughout evolution. Furthermore, collected evidences show that miR-223 expression is highly correlated with hematopoietic events and osteoclastogenesis throughout zebrafish development. In adults, zebrafish miR-223 tissue distribution mimics that of mice, with high levels of expression in the major hematopoietic organ, the head kidney. These results suggest that miR-

223 role in hematopoiesis has been maintained in zebrafish. Furthermore, validated targets of miR-223 in mammalian models were investigated and defined as putative targets in zebrafish, by *in silico* analysis. Our data compiles critical evidence showing that miR-223, which is highly conserved between species, appears to have also similar regulatory functions throughout evolution.

2.2. Introduction

MiRNAs are a conserved class of noncoding small RNAs, encoded in long primary transcripts (pri-miRNA) transcribed mostly by RNA polymerase II (Lee et al., 2004). Processing by the nuclear RNase III Drosha originates a miRNA precursor (pre-miRNA) which is further catalyzed in the cytoplasm by Dicer, generating a double-stranded 20-23 nt product (Davis and Hata, 2009; Du and Zamore, 2005). After loading into the RNA induced silencing complex (RISC), mature miRNAs guide the complex to target mRNAs and promote binding to complementary sequences in the 3' UTR, leading to translation inhibition or cleavage of RNA transcripts (Du and Zamore, 2005; Kim et al., 2009). As key regulators of gene expression, miRNAs have been implicated in a variety of developmental, physiological and pathological processes, including cell proliferation, apoptosis, differentiation and cell fate decisions (Erson and Petty, 2008; Fatica et al., 2008; Ivey and Srivastava, 2010; Wang et al., 2013b; Wienholds and Plasterk, 2005; Wu et al., 2012; Xiong et al., 2010). MiR-223 is a highly conserved miRNA known to be essential for normal myeloid cell differentiation (Chen et al., 2004; Johnnidis et al., 2008). In that sense, miR-223 was demonstrated to trigger granulocyte differentiation by targeting nuclear factor I/A (*NFIA*) and insulin-like growth factor 1 receptor (*IGF1R*) (Fazi et al., 2005; Lu et al., 2013), and to block erythrocytic differentiation by silencing LIM domain only 2 (rhombotin-like) (*LMO2*) (Felli et al., 2009). Furthermore, miR-223 was shown to be a key player in the maturation of granulocytes, through modulation of myocyte enhancer factor 2C (*MEF2C*) (Johnnidis et al., 2008) and in osteoclast differentiation by regulation of a *NFIA*/macrophage colony stimulating factor receptor-like (*M-CSFR*) mechanism (Sugatani and Hruska, 2009). Despite the recent progress towards elucidation of miR-223 function in

mammals, characterization of miR-223 regulatory network and function during development could help to clarify its hematopoietic and osteoclastogenic role in vertebrates and elucidate its mechanisms in human diseases, such as leukaemia and lymphoma. Zebrafish is a widely used model for studies of vertebrate development, gene function and human disease (Haffter et al., 1996; Lieschke and Currie, 2007). The rapid external embryonic development, transparency and survival of embryos without circulating blood cells for several days (Weinstein et al., 1996) and availability of mutants and transgenic lines (Sood and Liu, 2012) make zebrafish an ideal model to study hematopoiesis throughout development. In addition, zebrafish hematopoiesis presents anatomic, physiologic and genetic conservation with that of mammals (Carradice and Lieschke, 2008), which also experience two waves of hematopoiesis: primitive and definitive. Primitive hematopoiesis occurs in two phases: first, primitive macrophages arise from cephalic mesoderm and migrate onto the yolk ball; then, erythrocyte precursors develop in the intermediate cell mass (ICM) (Bertrand and Traver, 2009; Davidson and Zon, 2004; de Jong and Zon, 2005). Definitive hematopoiesis also occurs in two phases: first generating transient erythroid-myeloid progenitors (EMPs) in the posterior blood island (PBI) (Bertrand et al., 2007); and then producing hematopoietic stem cells (HSCs) in the AGM (aorta-gonad-mesonephros) region, which migrate to the caudal hematopoietic tissue (CHT) to support larval definitive hematopoiesis and to thymus and kidney to support adult definitive hematopoiesis (Bertrand and Traver, 2009; Jin et al., 2007; Paik and Zon, 2010). Despite all the similarities between zebrafish and mammalian hematopoiesis and the advantages of using this fish model to study this process, no data about miR-223 function is available in zebrafish.

In this work, we have investigated the possible functional conservation of miR-223 between zebrafish and mammalian models (human and/or mouse) by characterizing and comparing its gene organization, primary and secondary structures of the precursor and genomic context. Furthermore, we also explored the conservation of miR-223 spatial and temporal distribution and predicted targets. Our data shows that all miR-223 analysed features are generally

conserved between mammals and zebrafish, indicating that the zebrafish can be an excellent model to study miR-223 role in hematopoiesis throughout development.

2.3. Materials and Methods

2.3.1. Biological Material

Wild-type zebrafish were reared at 28.5ºC on a 14:10 hour light:dark cycle and zebrafish eggs obtained by natural spawning. Larvae were maintained and raised at standard conditions, as previously described (Westerfield, 2000). Individuals were collected randomly at regular intervals, from hatching to adult stages. Fish were euthanized with a lethal dose of MS-222 (Sigma) and either frozen in liquid nitrogen and preserved at -80ºC, or fixed in 4% buffered paraformaldehyde (PFA) at 4ºC for further processing.
Adult mice tissue samples were obtained from *Mus musculus* specimens maintained at the University of Algarve animal facilities.

2.3.2. Total RNA extraction

Total RNA was extracted following the Chomczynski and Sacchi method (Chomczynski and Sacchi, 1987). Total RNA samples were treated with RQ1 RNase-free DNase (Promega), according to manufacturer's instructions, and re-purified in phenol/chlorophorm/isoamyl alcohol (25:24:1) mixture. Total RNA was quantified by UV spectrophotometry (NanoDropND-1000) and its quality analysed in agarose gel electrophoresis.

2.3.3. Marathon library and molecular cloning of zebrafish miR-223 primary transcript

Zebrafish specimens (48 hours post fertilization (hpf), 20 days post fertilization (dpf), 25 dpf, 40 dpf, two adult males and two adult females) were collected for total RNA extraction (see previous section). RNA was then used to construct a Marathon^{TM} cDNA library (Clontech), following manufacturers' protocol. The 5' and 3' ends of zebrafish miR-223 were achieved by rapid

amplification of cDNA ends (RACE) using Advantage cDNA polymerase mix (Clontech) according to manufacturer's conditions. Specific forward and reverse primers (Dre pri-miR-223 Fw1 and 2, Rev1 and 2; listed in Supp. Table 2.1) were designed according to expressed sequence tag (EST) sequence available in National Center for Biotechnology Information (NCBI database) (http://www.ncbi.nlm.nih.gov) and combined with universal adapter primers (AP1 and AP2 universal primers; Supp. Table 2.1). Amplified PCR products were subsequently inserted into pCRII-TOPO (Invitrogen) and then cloned and further analysed by standard DNA sequencing.

2.3.4. Quantitative real-time PCR (qPCR) analysis

Total RNA was isolated from different adult tissues of zebrafish specimens (brain, heart, muscle, head kidney, branchial arches, skull and vertebrae) and mice specimens (ear, femur, calvaria, vertebrae, brain, muscle, heart, bone marrow). Regarding developmental stages, total RNA was extracted from a pool of up to twenty zebrafish larvae and juveniles at the following stages: 1 k-cell (approximately 3 hpf), 18 somites (approximately 16 hpf), 24, 31, 32, 33, 35 and 36 hpf, 2, 3, 4, 6, 15, 30, 45, 54, 60, 63, 69 and 81 dpf. Total RNA from one adult male and one adult female was also extracted.

For qPCR analysis of mature miRNAs, total RNA (1 µg) was polyadenylated and reverse-transcribed using the NCode miRNA First-Strand cDNA Synthesis kit (Invitrogen), according to manufacturer's instructions. PCR amplifications were performed using 2 µl of the reverse transcribed RNA (1:10 diluted), miRNA-specific primers and the NCODE qPCR kit (Invitrogen), according to manufacturer's instructions. Relative expression was determined through the ΔΔCt method (Livak and Schmittgen, 2001). Mature miRNA relative expression was normalized using U6 small nuclear RNA transcript. Primers and respective sequences are listed in Supp. Table 2.1.

2.3.5. In silico analysis

Precursor and mature miR-223 sequences from different vertebrate species were obtained from miRbase database release 20

(http://www.mirbase.org). EST and whole genome shotgun (WGS) sequences were obtained from NCBI database. Precursor sequences were aligned using Clustal Omega tool (http://www.ebi.ac.uk/Tools/msa/clustalo/) set for RNA and default parameters. This alignment was displayed as a logo using Weblogo facilities (http://weblogo.berkeley.edu/logo.cgi) and used to generate a consensus secondary structure using RNAalifold Web Server (http://rna.tbi.univie.ac.at/cgi-bin/RNAalifold.cgi). Pairwise sequence identity among different pre- and mature miR-223 sequences was determined using the Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/ident_sim.html). Values were calculated as percentage of identical nucleic acids over the total number of aligned nucleic acids. Individual structural analysis of miR-223 precursors was performed using Sfold software (http://sfold.wadsworth.org/cgi-bin/srna.pl). Finally, Ensembl genome database (http://www.ensembl.org/index.html) was used to identify the loci and the flanking regions of each mir-223 gene. Synteny was then established by comparing flanking genes of mir-223 from zebrafish and human. Genes were designated syntenic if one or more genes were conserved between zebrafish and human chromosomes, irrespective of orientation or order.

2.3.6. Prediction of miR-223 target transcripts

Pubmed database, available at NCBI (http://www.ncbi.nlm.nih.gov/pubmed/), was used to search for known targets of miR-223 in mammals. Then, zebrafish orthologs were retrieved from NCBI and 3´UTR of putative targets were fed to three different target prediction algorithms: i) TargetScanFish Release 6.2 (http://www.targetscan.org/fish_62/), based on conserved sites in mRNA 3´UTR matching the seed region of miRNA, and ranking according to combinatorial scores of number of sites, type and context (Garcia, et al. 2011; Grimson et al., 2007; Lewis et al., 2005; Ulitsky et al., 2012); ii) PITA algorithm (http://genie.weizmann.ac.il/pubs/mir07/index.html), which computes the difference between free energy associated to miRNA-target duplex and free energy associated to unpairing the target to make it accessible to the miRNA

(Kertesz et al., 2007) and iii) RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html), based on the minimum free energy of hybridization of a long and a short RNA (Rehmsmeier et al., 2004). Putative targets were considered whenever the same binding sites were predicted by at least 2 of the algorithms used.

2.4. Results

We analysed whether zebrafish could be a valid model to study miR-223 function, and if data available in other models, namely in mammals, could be further confirmed and, more importantly, extended using zebrafish as a model. For that, conservation of miR-223 was investigated from different perspectives: from sequence to structure, from gene organization to genomic context, and from levels of expression to mRNA targets.

2.4.1. Identification of zebrafish miR-223 primary transcript and gene structure

Human and zebrafish pre-miR-223 sequences were initially collected from miRBase database. However, as for other pre-miRNAs, pre-miR-223 constitutes only a partial sequence of the full-length transcript. In order to identify the sequences of both the complete transcripts and possible variants, human and zebrafish pre-miR-223 were used as queries in BLAST tool from NCBI database to search for non-annotated cDNA fragments. While searching for human sequences we retrieved two complete mRNA sequences (accession numbers DQ680071 and DQ680072), corresponding to different variants of full-length miR-223 transcript, and possibly resulting from alternative splicing events (Fig. 2.1). However, in zebrafish, only a single EST (accession number EB991492) was found, matching pre-miR-223 sequence. Since the zebrafish cDNA sequence was partial, it was used as template to design specific primers and identify the complete transcript. After RACE-PCR amplification, cDNA cloning and standard sequencing, a full-length sequence spanning 1146 base pairs (bp) and matching zebrafish miR-223 was identified and submitted to NCBI database (accession number KJ634046). Then, human and zebrafish

miR-223 transcripts sequences were used to search genomic sequence available in Ensembl database. After comparing and aligning genomic and transcript sequences using Splign tool available in NCBI, human and zebrafish mir-223 gene structures were determined (Fig. 2.1). According to this analysis, the zebrafish mir-223 gene is located in chromosome 5 (Chr 5, - strand), it spans approximately 11.8 kilobase pairs (kb) and is composed of 3 exons. Pre-miR-223 is inserted in the third exon and, so far, only one variant has been identified (Fig. 2.1). This structure is maintained in human (Fig. 2.1), where the corresponding gene is also composed of 3 exons spanning 5 kb and located in the X chromosome (sex chromosome; Chr X, + strand). As mentioned before, the two human cDNA fragments appear to result from alternative splicing events and structure analysis suggests alternative splicing of exon 2, which is either present or absent in human miR-223 transcript.

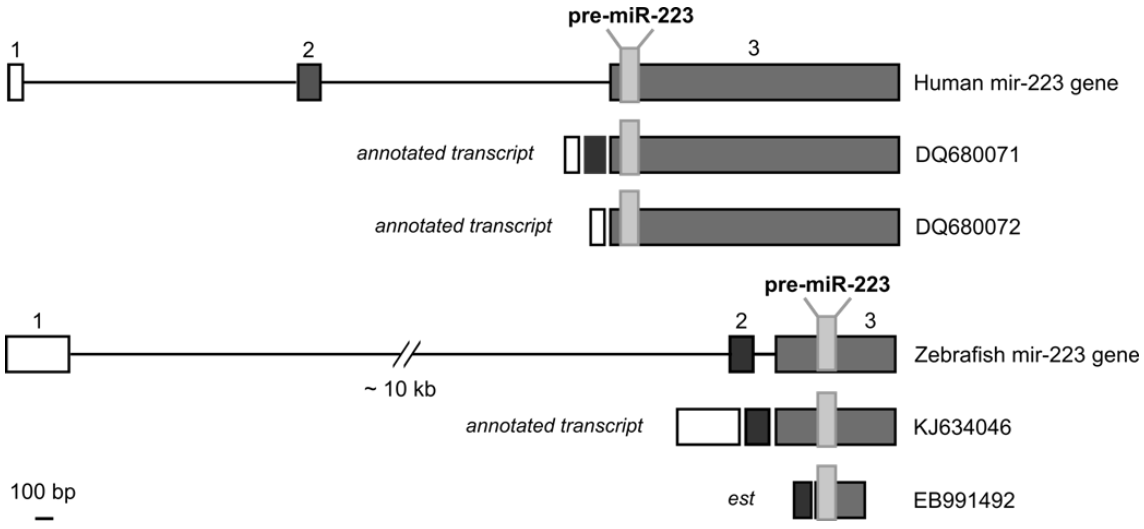

Figure. 2.1. Structural organization of mir-223 gene. A schematic representation of human and zebrafish mir-223 genes located, respectively, in Chr X and 5, is presented. Bellow human gene structure, 2 annotated transcript variants are represented (accession numbers DQ680071 and DQ680072). Bellow zebrafish gene structure, the primary transcript here cloned and annotated (accession number KJ634046) and an EST (accession number EB991492) are schematized. Boxes represent exons 1, 2 and 3 indicated in white, black and dark grey respectively. Pre-miR-223 is indicated in a light grey box, within exon 3.

2.4.2. Genomic context of zebrafish and human mir-223 genes

The genomic context of zebrafish mir-223 gene was analyzed and compared to its human counterpart. According to information available in Ensembl and miRbase, the zebrafish mir-223 gene is located in the negative strand of Chr 5, at position 24 Mb (5: 24017735-24017833 [-]), while the human mir-223 gene is located in the positive strand of Chr X at position 65 Mb (chrXq21.1: 65238712-65238821 [+]). Zebrafish Chr 5 spans approximately 75.68 Mb and contains 427 non-coding genes, whereas human Chr X spans approximately 155.27 Mb and accounts for 735 non-coding genes. According to miRbase, a total of 23 and 118 miRNAs are assigned to zebrafish Chr 5 and human Chr X, respectively. A more detailed analysis of the genomic context of mir-223 gene revealed strong conservation between both species. In this regard, the upstream flanking regions of both human and zebrafish mir-223 loci are composed by a group of eight common genes presenting a similar spatial organization: moesin (*MSN*), lethal in the *a*bsence of *S*sd1 (LAS1)-like (*LAS1L*), zinc finger CCCH-type containing 12B (*ZC3H12B*), zinc finger C4H2 domain containing (*ZC4H2*), myotubularin related protein 8 (*MTMR8*), ankyrin repeat and SOCS box containing 12 (*ASB12*), family with sequence similarity 123B (*FAM123B*) and Cdc42 guanine nucleotide exchange factor (GEF) 9 (*ARHGEF9*) (Fig. 2.2). Downstream of mir-223 gene, the order of gene loci was much less preserved, although several genes were still conserved between the two species. Conserved genes downstream mir-223 include: non-POU domain containing, octamer-binding (*NONO*), uracil phosphoribosyltransferase (*FUR1*) homolog (*UPRT*), bromodomain and WD repeat domain containing 3 (*BRWD3*) and KIAA2022 (Fig. 2.2).

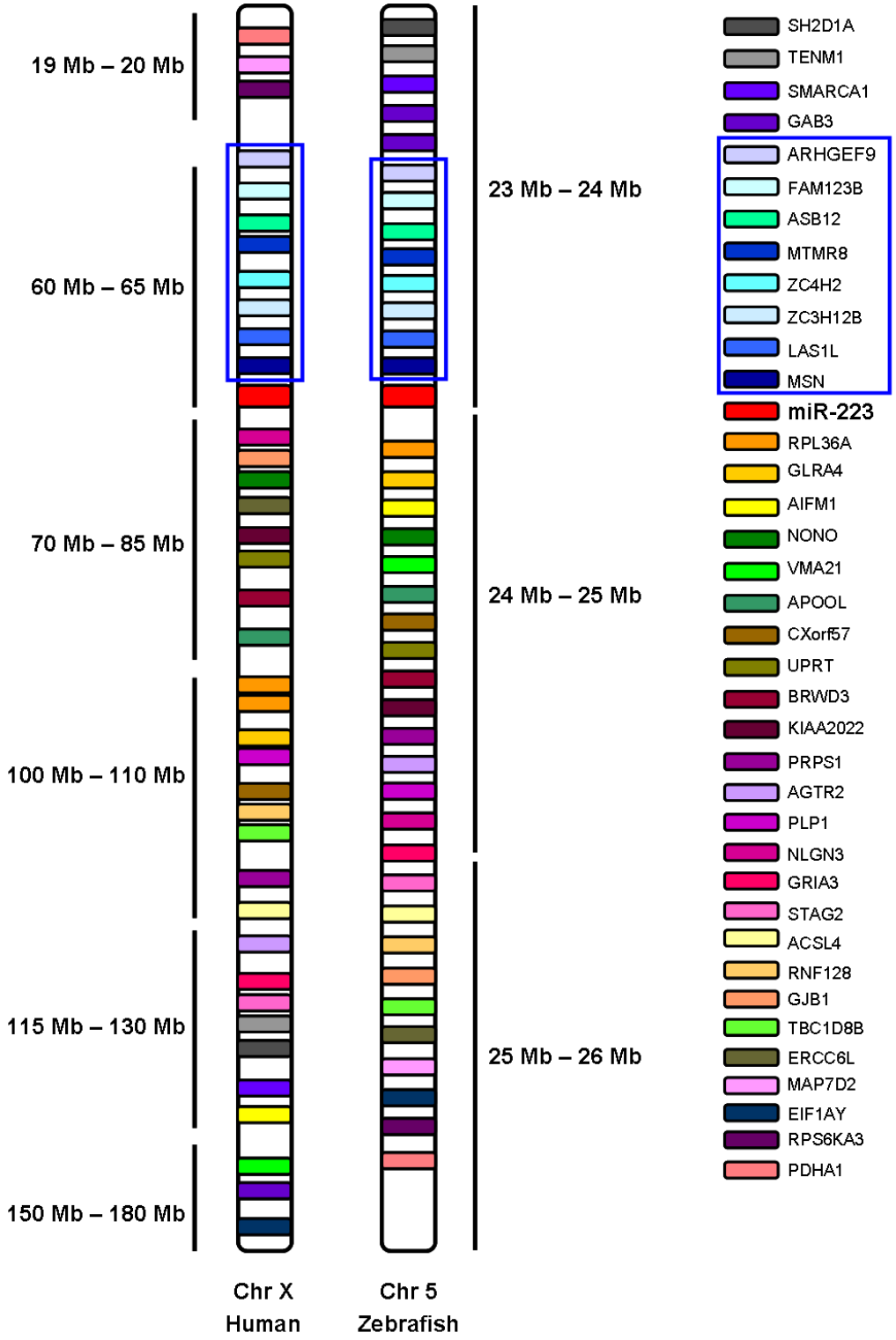

Figure. 2.2 Schematic representation of the genomic context of human and zebrafish mir-223 genes using data from the Ensembl project. The physical localization of the genes present in the vicinity of mir-223 gene is indicated for human and zebrafish. Genes ID are listed at the right with mir-223 indicated in bold. Eight genes conserved in the same order are highlighted with a blue box in the chromosomes of both species and in the IDs list. Chr – chromosome.

2.4.3. Precursor of miR-223 contains highly conserved domains in vertebrates

In order to further investigate the conservation of miR-223 among different vertebrate species (mammals, amphibian, birds, reptiles and teleost fish), the corresponding sequences of its precursor (pre-miR-223) were collected from miRbase and their pairwise identities determined. According to this analysis, pre-miR-223 displayed different homologies among different taxa, varying from 80% (*Saguinus labiatus* versus *Gallus gallus*) to 47.66% (*Ornithorhynchus anatinus* versus zebrafish), clearly showing higher sequence conservation among mammalian species. Regarding zebrafish pre-miR-223, sequence identity ranged from 57 to 64% when comparing to mammalian species (excluding *O. anatinus*), from 51 to 57% when comparing to amphibian/birds/reptiles, and from 55 to 69% when comparing to other teleost fish (Supplementary Fig. 2.1a). These data suggested a similar conservation between zebrafish and other species from the same taxa (i.e. teleost fish), and also between zebrafish and species from other taxa (e.g. mammalian). To further complement this analysis, additional sequences were collected from EST and WGS available in NCBI, thus compensating the poor availability of sequences in certain taxonomic groups (e.g. birds and teleost fish). In total, 46 miR-223 precursor sequences were collected, i.e. 24 sequences previously annotated / available in miRbase, and 22 new sequences that were identified in the present study. For the latter, hairpin precursors were inferred based on: i) nucleotide, size and position similarity, when compared with species from the same taxa; and ii) hairpin minimum free energy. Then, precursor sequences were aligned using Clustal Omega and used to generate logos using WebLogo (Fig. 2.3a). Finally, a consensus secondary structure based on the previous alignments (Fig. 2.3b) and individual secondary structures (Fig. 2.4) were predicted using RNAalifold and Sfold, respectively. This analysis enabled us to conclude that some domains of miR-223 precursor sequence are generally conserved among vertebrates, while others are less conserved and therefore are specific of certain taxa. Not surprisingly, the most conserved domain corresponded to consensus mature miR-223 sequence (5’ –

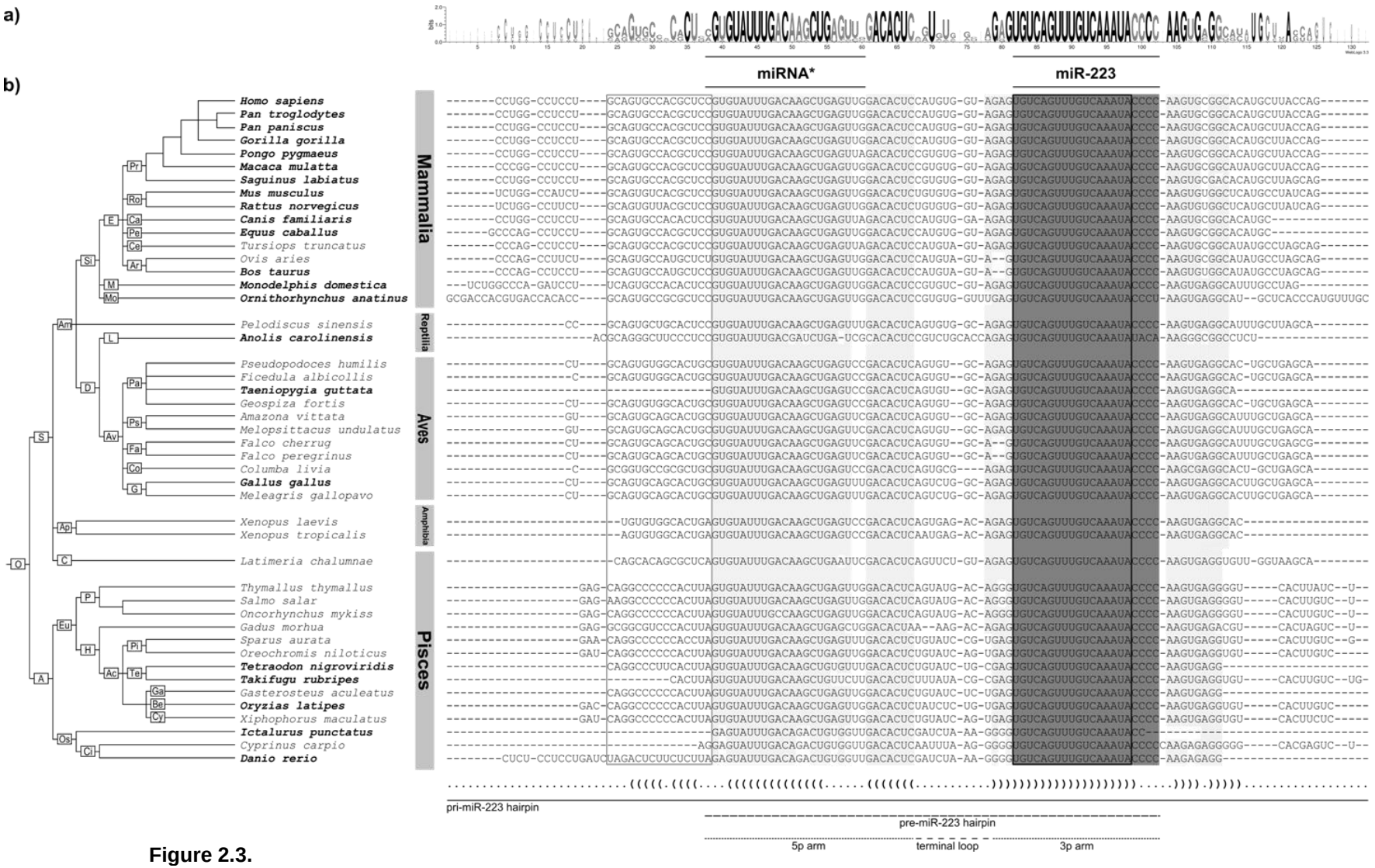
a)
b)
miRNA*
miR-223
Homo sapiens
Pan troglodytes
Pan paniscus
Gorilla gorilla
Pongo pygmaeus
Macaca mulatta
Saguinus labiatus
Mus musculus
Rattus norvegicus
Canis familiaris
Equus caballus
Tursiops truncatus
Ovis aries
Bos taurus
Monodelphis domestica
Ornithorhynchus anatinus
Pelodiscus sinensis
Anolis carolinensis
Pseudopodoces humilis
Ficedula albicollis
Taeniopygia guttata
Geospiza fortis
Amazona vittata
Melopsittacus undulatus
Falco cherrug
Falco peregrinus
Columba livia
Gallus gallus
Meleagris gallopavo
Xenopus laevis
Xenopus tropicalis
Latimeria chalumnae
Thymallus thymallus
Salmo salar
Oncorhynchus mykiss
Gadus morhua
Sparus aurata
Oreochromis niloticus
Tetraodon nigroviridis
Takifugu rubripes
Gasterosteus aculeatus
Oryzias latipes
Xiphophorus maculatus
Ictalurus punctatus
Cyprinus carpio
Danio rerio
Mammalia
Reptilia
Aves
Amphibia
Pisces
pri-miR-223 hairpin
pre-miR-223 hairpin
5p arm
terminal loop
3p arm

Figure 2.3.

Figure 2.3. Conserved features of miR-223 hairpin among vertebrate species. a) Alignment of predicted pre-miR-223 from 46 species using WebLogo. Sequence logos are presented as a graphical display where the height of each letter is made proportional to its frequency in each position and black letters in the logo indicates nucleotides that are 100% conserved. **b)** Mir-223 hairpin alignment using Clustal Omega is displayed together with taxonomic tree (left) and consensus secondary structure in dot-bracket notation (bottom, where dots and brackets represent unpaired and paired bases, respectively). Mature miR-223 sequence is highlighted in dark grey with the first 17 nucleotides, 100% conserved, enclosed in a box. Other conserved domains among species are highlighted in soft grey. A domain only conserved within the same group of species, preceding star strand, is indicated with a grey box. Consensus boundaries of pre-miR-223 are indicated as dashed lines at the bottom. Species corresponding to sequences retrieved from miRbase are indicated in bold. Taxonomic groups investigated were: Actinopterygii (A), Acanthopterygii (Ac), Amniota (Am), Amphibia (Ap), Artiodactyla (Ar), Aves (Av), Beliniformes (Be), Coelacanthimorpha (C), Carnívora (Ca), Cetacea (Ce), Cypriniformes (Ci), Columbiformes (Co), Cyprinodontiformes (Cy), Diapsida (D), Eutheria (E), Euteleostei (Eu), Falconiformes (Fa), Galliformes (G), Gasterosteiformes (Ga), Holacanthopterygii (H), Lepidosauria (L), Marsupialia (M), Monotremata (Mo), Osteichthyes (O), Ostariophysi (Os), Protacanthopterygii (P), Passeriformes (Pa), Perciformes (Pe), Perissodactyla (Pi), Primates (Pr), Psittaciformes (Ps), Rodentia (Ro), Sarcopterygii (S), Synapsida (Si), Testudines (T), Tetraodontiformes (Te).

UGUCAGUUUGUCAAAUACCCC – 3') (Fig. 2.3 and Supplementary Fig. 2.1b). This conservation is more notorious in the 5' end of the sequence (100% conservation in nucleotides 1 to 17; box in Fig. 2.3b), which should be related to functional aspects, namely specific interactions with target transcripts, as previously described (Pillai, 2005). Nevertheless, three species from different taxa showed important differences in the 3' end of the mature miR-223 (up to 3 different nucleotides), which could be related to evolutionary divergence of species. For instance, *O. anatinus,* which belongs to the monotremata order, diverged in 1 nucleotide (C-T) in the 3' end; *A. carolinensis*, a reptile, diverged from remaining species analysed by specific changes in the 19th (C-A) and 21st (C-A) positions of mature miR-223; *Ictalurus punctatus* lacked 2 nucleotides in the 3' end of the mature sequence. In the last example, it remains unclear whether this unique feature represents a true divergence or if corresponds to an incomplete sequence. Another important conserved domain is the miR-223 star strand (although miR-223 star is only described in 5 species: *H. sapiens, M. musculus*, *R. norvegicus*, *O. anatinus* and *A. carolinensis*, miR-223 star strand will be here referred as the sequence corresponding to the putative miR-223 star) (Fig. 2.3). Nevertheless, in miR-223 star strand some degree of divergence was obvious, especially among actinopterygii group, where 7 out of 14 species contained up to 5 different nucleotides. Finally, high conservation

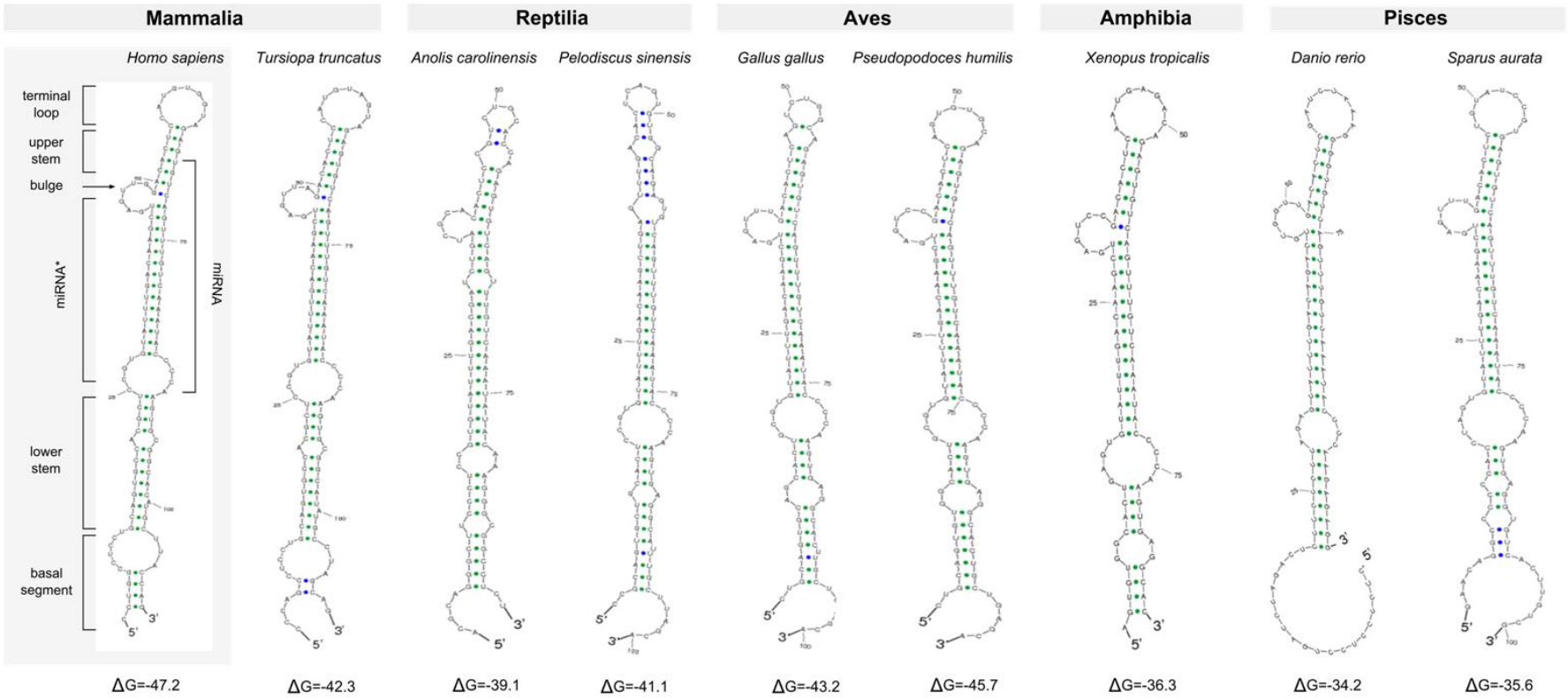

Figure 2.4. Pri-miR-223 secondary structure conservation among vertebrates (predicted by Sfold). Two species representative of each taxonomic group, one retrieved from miRbase (*Homo sapiens*, *Anolis carolinensis*, *Gallus gallus*, *Xenopus tropicalis* and *Danio rerio*) and another described / identified here (*Tursiopa truncates*, *Pelodiscus sinensis*, *Pseudopodoces humilis*, *Sparus aurata*), were selected to illustrate conservation of predicted stem loops. Important features of the stem loop are indicated in the *Homo sapiens* pri-miR-223 structure. ΔG represents free energy at a folding temperature of 37ºC.

was also evident among flanking sequences of mature miR-223 at both 5' (consensus GAG) and 3' ends (consensus AAGUG), as also observed for the sequence flanking the 3' end of star strand sequence (consensus GACACUC) (Fig. 2.3).

Regarding less conserved domains, relatively high conservation was still evident within the same taxa or in some cases among different taxa. An example of this is the flanking sequence preceding the star strand, which contains at least 11 conserved nucleotides within species of the same group: mammalian, birds, amphibian and teleosts (first group of shaded columns on Fig. 2.3b). Concerning analysis of miR-223 precursor secondary structures, all analysed sequences resulted in prediction of stem-loops (Fig. 2.4), which is a characteristic feature of pre-miRNAs. Some motifs of the predicted stem-loops were highly conserved, including: i) terminal loop, ii) bulge in 5´ arm and iii) internal loop containing the last nucleotides of 3´ end of mature miR-223 (Fig. 2.4). This conservation was confirmed by consensus secondary structure illustrated in Fig. 2.3b. A common feature in all analysed species was that mature miR-223 was maintained in the 3p arm of all stem-loops (Figs. 2.3 and 2.4).

2.4.4. Zebrafish miR-223 expression analysis

In mammals, miR-223 was clearly associated with hematopoiesis (reviewed by (Haneklaus et al., 2013)) and osteoclastogenesis (Sugatani and Hruska, 2007, 2009). In zebrafish, crucial events of hematopoiesis were previously described between 10 hpf and 6 dpf, (Bertrand et al., 2007, 2008; Paik and Zon, 2010; Sood and Liu, 2012), while osteoclastogenesis was shown to occur from 20 dpf until adulthood (Witten et al., 2001). qPCR analysis of miR-223 from blastula to larval stages of zebrafish development revealed that miR-223 expression is strongly up-regulated from 31 to 35 hpf (peak at 32 hpf) and 4 to 6 dpf, while it is only mildly increased at 24 hpf, 36 hpf, 2 dpf and 3 dpf (Fig. 2.5). In juveniles, i.e. after 30 dpf, miR-223 levels of expression were consistently elevated, with a peak being reached at approximately 60 dpf (Fig. 2.5). In general, miR-223 up-regulation was maintained until adulthood, and both male and female showed

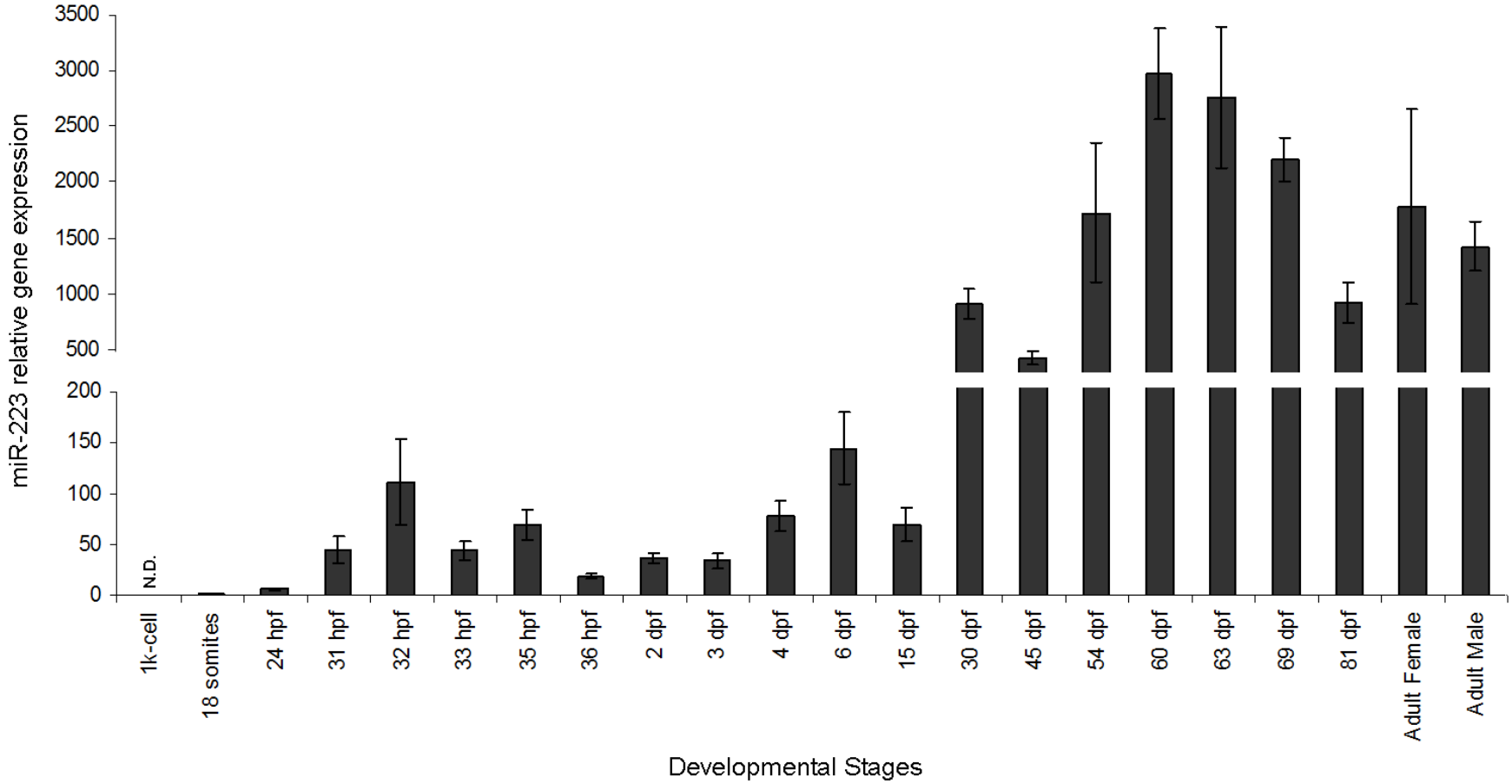

Figure. 2.5. Analysis of mature miR-223 expression levels during development of zebrafish. Levels of miR-223 gene expression were measured by qPCR using RNA samples from different stages of zebrafish development, normalized to levels of zebrafish U6 small RNA and using 18 somites (18 S) as reference sample. Values are the mean of at least 3 independent replicates; *hpf* indicates hours post fertilization; *dpf* indicates days post fertilization, *N.D.* indicates non-detected. Gap in the *y* axis separates two different scales.

similar levels of expression (Fig. 2.5). In adult zebrafish, miR-223 expression was investigated in calcified (i.e. branchial arches, skull and vertebrae) and soft (i.e. brain, muscle, heart, and head kidney) tissues (Fig. 2.6a). Although miR-223 expression was detected in all analysed tissues, it was remarkably higher in the head kidney (Fig. 2.6a), a well-described hematopoietic organ in zebrafish, equivalent to bone marrow in mammals (Paik and Zon, 2010; Sood and Liu, 2012). Relatively high levels of expression were also observed in vertebrae, muscle, and heart. Since miR-223 has been related with differentiation of hematopoietic lineages, higher levels of expression in heart or muscle could be associated to their high degree of vascularization (probably due to circulation of immature cells). Alternatively, miR-223 could be associated to specific functions of these organs. In mammals, miR-223 expression is also known to be increased at sites of hematopoiesis (Chen et al., 2004). Here, miR-223 expression was also analysed in several tissues from adult mice: 4 calcified tissues, i.e. ear, femur, calvaria and vertebrae, and 4 soft tissues, i.e. brain, muscle, heart and bone marrow. The obtained results not only confirmed previous studies (Chen et al., 2004), but also showed that the miR-223 pattern of expression in adult mice (Fig. 2.6b) resembles that observed in zebrafish, i.e. presence of miR-223 in all analysed tissues, and highest expression clearly associated to major sites of hematopoiesis (here represented by bone marrow). High expression levels were also observed in femur, calvaria, vertebrae, muscle and heart (Fig. 2.6b). Like in zebrafish, brain and cartilaginous tissues in mice were the ones presenting lower levels of miR-223 expression (Fig. 2.6a and b).

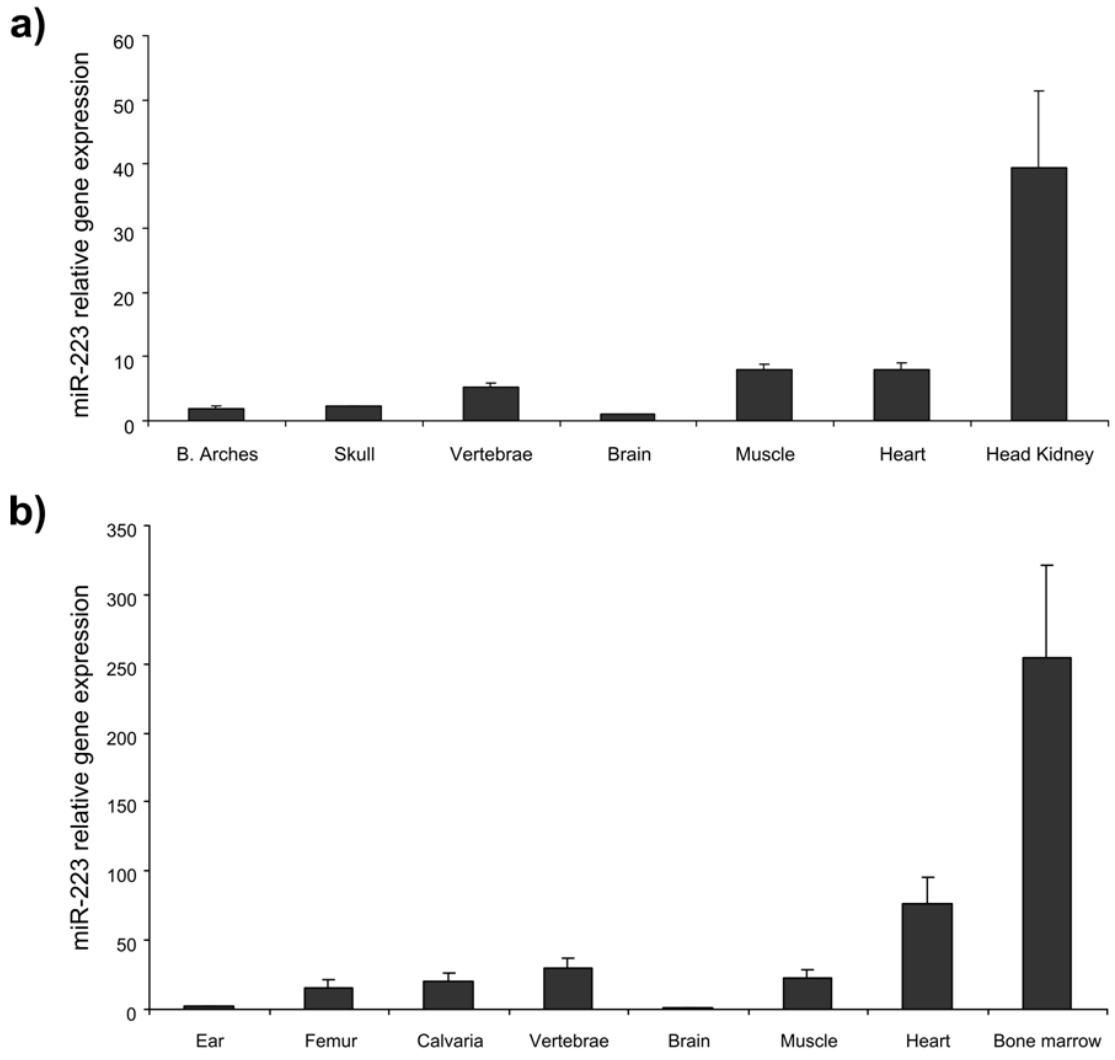

Figure 2.6. Analysis of mature miR-223 expression levels in zebrafish (a) and mouse **(b)** adult tissues. Levels of miR-223 gene expression measured by qPCR in zebrafish and mouse tissues were normalized to levels of respective U6 small RNA and using brain as reference sample. Values are the mean of at least 3 independent replicates.

2.4.5. Identification of zebrafish putative miR-223 targets

In order to further complement previous data showing miR-223 functional conservation between zebrafish and mammals, miR-223 targets were investigated in the literature through extensive search of Pubmed database, using the following keywords: miR-223 and target (last checked in November 18, 2013). Each miRNA can regulate hundreds of genes (Pillai, 2005; Rajewsky, 2006), and accordingly, miR-223 was previously demonstrated to

regulate several mammalian mRNAs (reviewed by Haneklaus et al., 2013). Although our search retrieved a total of 92 reports, 56 publications were excluded from this analysis due to lack of one or more of the following inclusion criteria: i) presence of respective ortholog in zebrafish; ii) knowledge of full-length cDNA sequence in zebrafish; iii) association of targets to mammalian hematopoiesis (miR-223 role was shown to be mainly associated to this process in mammals). The remaining studies, 36 in total, corresponded to a total of 9 miR-223 targets identified in mammalian systems (Table 2.1). The zebrafish orthologs of these targets were searched and retrieved from NCBI database and analysed for putative miR-223 binding sites using three different bioinformatic tools (Table 2.2): i) TargetScanFish, ii) PITA algorithm and iii) RNAhybrid. Potential binding sites were considered if identified by at least two algorithms. In Table 2.1, miR-223 targets identified in mammalian systems were listed along with the respective reports, whereas Table 2.2 resumes the *in silico* analysis of corresponding zebrafish ortholog genes. According to this analysis, both zebrafish orthologs of RAS p21 protein activator (*RASA1*) (rasa1a and *rasa1b*) and stathmin 1 *STMN1* (*stmn1a* and *stmn1b*) contained putative binding sites for miR-223, while only one zebrafish ortholog for *IGF1R* and *MEF2C* fit the criteria established in this study (Table 2.2). While predicting these targets, it was common to find one or more paralogs also predicted as miR-223 targets (data not shown) in zebrafish (7 of the 9 targets), which could be related to genome-wide duplication events in zebrafish lineage that occurred 300-500 million years ago (Meyer and Schartl, 1999; Taylor et al., 2003). Although paralog genes may not share exactly the same function, it is likely that some of these genes might complement each other, or their functions partially overlap, as previously described for other paralogs (Boucherat et al., 2013; Solomon and Fritz, 2002; Yuan et al., 2010).

Gene	Previous Evidences for miR-223/Target interaction in Mammalian Systems
NFIA nuclear factor I/A	miR-223 represses translation of NFI-A favouring granulocytic differentiaton [14]. miR-223 regulation of NFI-A stimulates osteoclast differentiation and function by favouring M-CSFR expression [17].
RASA1 RAS p21 protein activator 1	Although Rasa1 was not the functional target in the system studied, Rasa1 is a direct target of miR-223 [74].
E2F1 E2F transcription factor 1	miR-223 is a master regulator of myeloid cell proliferation interlinked with E2F1 in a mutual negative feedback loop [80].
FBXW7 F-box and WD repeat domain-containing 7	High expression of miR-223 has an adverse impact on the survival of ESCC patients by repression of FBXW7 [81]. TAL1-mediated up-regulation of miR-223 promotes the malignant phenotype in T-ALL through repression of the FBXW7 tumor suppressor [82].
FOXO1 forkhead box O1	Overexpression of miR-223 lead to down-regulation of FOXO1 and inhibition of cell proliferation [11].
IGF1R insulin-like growth factor 1 receptor	Regulation of IGF-1R represents a potential mechanism by which miR-223 controls granulocyte function [13]. miR-223 supressed HeLa cells proliferation by targeting IGF-1R [74]. miR-223 regulates eosinophil differentiation probably trough IGF-1R [15].
MEF2C myocyte enhancer factor 2C	miR-223 targets Mef2c in myeloid progenitors inhibiting their proliferation and granulocyte function [13]. miR-223 is up-regulated in calcifying VSMCs and its overexpression promotes VSMCs proliferation and downregulates Mef2c and RhoB [87].
STMN1 stathmin 1	STMN1 is a downstream target of miR-223 in hepatocellular carcinoma [83]. STMN1 is a putative target of miR-223 in gastric cancer cells [84].
LMO2 LIM domain only 2 (rhombotin-like 1)	miR-223 reversibly regulates erythroid and megakaryocytic differentiation of K562 cells by targeting LMO2 [61]. Enforced expression of miR-223 impairs differentiation of erythroid cells by repressing LMO2 [16].

Table 2.1. Mammalian target genes of miR-223 analysed in this study.

Gene	Target prediction in Zebrafish			
	Aces. Num.	Seed match	Predicted by	Biological process
NFIA nuclear factor I/A	XM_005161709.1	1922 - 1928	TargetScan, PITA, RNAhybrid	DNA replication, regulation of transcription, DNA-dependent transcription, DNA-templated
RASA1 RAS p21 protein activator 1	rasa1a (XM_001341973.5) rasa1b (XM_001921687.4)	264-270 329-335	TargetScan, PITA, RNAhybrid	regulation of small GTPase mediated signal transduction
E2F1 E2F transcription factor 1	XM_005174533.1	39 - 46 1705 - 1713	TargetScan, PITA, RNAhybrid	unknown
FBXW7 F-box and WD repeat domain-containing 7	XM_005170948.1	242 - 249	TargetScan, PITA, RNAhybrid	negative regulation of Notch signaling pathway angiogenesis regulation of transcription, DNA-dependent transcription, DNA-templated
FOXO1 forkhead box O1	foxo1a (BX649258.11)	3450- 3458 3468 - 3474	PITA, RNAhybrid TargetScan, PITA, RNAhybrid	
IGF1R insulin-like growth factor 1 receptor	igf1ra (BX470160.9)	585 - 592 2530 - 2537	PITA, RNAhybrid TargetScan, PITA	anterior/posterior pattern specification; heart morphogenesis and development; embryo development; IGF receptor signaling pathway
MEF2C myocyte enhancer factor 2C	mef2cb (BX465834.20)	1355 - 1361	PITA, RNAhybrid	cardiac muscle cell development and differentiation heart formation and development regulation of transcription, DNA-dependent
STMN1 stathmin 1	stmn1a (NM_203401.1) stmn1b (NM_001017850.1)	354 - 360 746 - 752	PITA, RNAhybrid	regulation of microtubule polymerization
LMO2 LIM domain only 2 (rhombotin-like 1)	AF191560.1	404 - 411	PITA, RNAhybrid	blood vessel development; embryonic hemopoiesis erythrocyte differentiation multicellular organismal development

Table 2.2. Resume of *in silico* analysis of zebrafish miR-223 putative target genes. Position of seed match refers to counting from first nucleotide after stop codon.

Regarding gene function, zebrafish predicted targets were associated to two main molecular functions: regulation of transcription (*nfia*, *mef2cb*, *foxo1a* – forkhead box O1a) and of different signalling pathways (F-box and WD repeat domain containing 7, E3 ubiquitin protein ligase (*fbxw7*), *igf1ra*). The following biological processes were associated to these genes: i) cardiovascular system formation (*igf1ra*, *mef2cb*, *lmo2*, *fbxw7*, *rasa1*); ii) hematopoiesis (*lmo2*); and iii) embryo development (*igf1ra*) (Table 2.2). Unfortunately, zebrafish ortholog of E2F transcription factor 1 (*E2F1*) has not been functionally annotated yet.

Altogether, the available data suggest that miR-223 targets and function may have been conserved from zebrafish to mammals.

2.5. Discussion

This work focused on exploring the functional conservation of miR-223 throughout evolution, from mammals to zebrafish. MiR-223 gene structure, primary and secondary structures of its precursor, its genomic context, spatial-temporal expression and putative targets were characterized in zebrafish and compared to those from human and/or mouse models. Our results indicate that zebrafish has an orthologue to human miR-223 gene, and expression and analysis of predicted targets point towards a functional conservation in hematopoiesis and osteoclastogenesis.

2.5.1. miR-223 is conserved throughout evolution: insights from gene and precursor analysis

In order to investigate the putative miR-223 conservation in vertebrates, the zebrafish full-length primary transcript of miR-223 was identified and mapped to the zebrafish genome. Like in other vertebrates, e.g. human and mouse (Fukao et al., 2007), zebrafish mir-223 gene is organized in three exons and its precursor sequence is contained in the third exon, demonstrating a structural genomic conservation. Nonetheless, human and mouse mir-223 genes are more compact than that of zebrafish, with two alternative splicing forms already described (Fukao et al., 2007), while only one transcript was so far identified in zebrafish through this study. The question of whether this

transcript was in fact an ortholog of the human miR-223 led to a comparison of the genomic context between zebrafish (Chr 5) and human (Chr X). Gene synteny analysis revealed a block of 8 genes upstream of mir-223 locus that was preserved in the same order between the two species, whereas downstream mir-223 the order of syntenic genes was less preserved. Therefore, gene synteny analysis, a valuable tool to determine orthologs (Postlethwait, 2000), confirmed that zebrafish mir-223 is likely to be a true ortholog of the corresponding human gene. To further investigate the conservation of miR-223, important features such as its pre-miRNA primary (Auyeung et al., 2013; Saetrom et al., 2006) and secondary structures (Gu et al., 2012; Han et al., 2006; Park et al., 2011; Wostenberg et al., 2012; Zeng and Cullen, 2005; Zeng et al., 2005b; Zhang and Zeng, 2010) were analyzed in a broad group of species following established guidelines. While nucleotide sequence identity was less than 70% between zebrafish and other vertebrate species, sequence alignment showed a remarkable conservation of the mature miR-223. In particular, the seed region, which is a key element in target recognition and translation inhibition (Doench and Sharp, 2004), was 100% preserved, suggesting a conservation of miR-223 putative targets and most likely of its function among vertebrates. Furthermore, the flanking sequences of both mature and star miR-223 were highly preserved, which might indicate that miR-223 processing is also conserved. This is further supported by the conservation of secondary structures in the miR-223 hairpin: i) a terminal loop, which is critical for Drosha and Dicer optimal processing, and contributes to determine the cleavage site by the distance to the loop (Gu et al., 2012; Zeng et al., 2005b; Zhang and Zeng, 2010); ii) single-stranded extensions on the pri-miRNA hairpin in the basal segment, which are vital for DGCR8 binding and distance counting for Drosha cleavage (Han et al., 2006; Zeng and Cullen, 2005; Zeng et al., 2005b); iii) 2 helix turns (~22 nt) that encode the miRNA:miRNA* duplex; and iv) 1 helix turn (~11 nt) of the lower stem, which is also important for processing (Han et al., 2006; Zeng et al., 2005b). Although the precise role of the various miRNA structures is not yet fully understood,

maintenance of these features provides further evidence for conservation of miR-223 processing.

In general, structural information pointed towards a notable conservation of miR-223 throughout evolution, raising the hypothesis that its function could also be maintained.

2.5.2. MiR-223 expression is correlated with hematopoiesis in zebrafish

In mammalian models, miR-223 is known to play critical roles in hematopoiesis, promoting granulocyte differentiation (Fazi et al., 2005; Fukao et al., 2007) and suppressing erythrocytic differentiation (Yuan et al., 2009a). To shed some light into the putative functions of miR-223 in other vertebrates, the expression pattern of this miRNA was investigated in zebrafish. According to our data, zebrafish miR-223 was up-regulated between 31 and 36 hpf, which could be related in particular with two important hematopoietic events: i) formation of committed erythromyeloid progenitors in the PBI, known to occur between 24 and 48 hpf (Bertrand et al., 2007); and ii) the formation of hematopoietic precursors in the AGM, occurring from 30-36 hpf (Bertrand et al., 2008). Furthermore, miR-223 was also up-regulated between 4 and 6 dpf, and from 30 dpf to adulthood, which could be associated, respectively, to the seeding of the head kidney by HSCs and to definitive hematopoiesis support (Bertrand and Traver, 2009; Cumano and Godin, 2007; de Jong and Zon, 2005; Paik and Zon, 2010; Sood and Liu, 2012). The finding that miR-223 was highly expressed in the primary hematopoietic organs of both zebrafish and mouse further supported its putative role in hematopoiesis and emphasized the zebrafish usefulness for elucidation of miR-223 function. In addition to hematopoiesis, a 10-fold induction of miR-223 expression after 30 dpf might as well be related to osteoclastogenesis and bone remodeling, which in zebrafish normally occur from 20-30 dpf onwards (Witten and Huysseune, 2009b; Witten et al., 2001). Although osteoclasts derive from the hematopoietic lineage, the molecular pathway(s) involved in this process are still not completely elucidated in mammals or zebrafish. However, miR-223 was previously shown to be a key factor in osteoclast differentiation in mammals, being differentially expressed

during the different phases of osteoclast differentiation (Kagiya and Nakamura, 2013; Shibuya et al., 2013; Sugatani and Hruska, 2007, 2009). Because of its similarities with mammals in terms of gene structure and targets, zebrafish might be an important model to clarify the exact function of miR-223 microRNA in osteoclastogenesis. In that sense, transgenic lines that allow *in vivo* imaging of osteoclasts maturation and migration (as already available in medaka (Chatani et al., 2011)) could be crossed with other lines, in which miR-223 promoter could drive expression of a fluorescent reporter gene thus providing relevant *in vivo* tools to further understand miR-223 function in vertebrates.

2.5.3. Evidences for conservation of miR-223 targets and regulatory functions in mammals and zebrafish

In general, in order to fully elucidate miRNAs functions, it is essential to identify their target transcripts. In mammals, the miR-223 major function appears to be the regulation of hematopoietic cell fate speciation and differentiation. Accordingly, several of the previously validated miR-223 target transcripts in mammalian systems were related to hematopoiesis. In this study, prediction of miR-223 putative targets in zebrafish strongly suggested once more an evolutionary conservation of its function in vertebrates. For instance, *LMO2* has a well-known and conserved function in hematopoiesis and is required for primitive and definitive hematopoiesis and for angiogenesis in mammals and zebrafish (Patterson et al., 2007; Wang et al., 2008; Yamada et al., 1998, 2000; Zhu et al., 2005). Furthermore, it was shown to be down-regulated by miR-223 in humans, suppressing differentiation of erythroid cells (Felli et al., 2009; Yuan et al., 2009a) and increasing megakaryocytic differentiation (Yuan et al., 2009a). *NFIA* is another gene that has been shown to be down-regulated by miR-223 to promote granulocytic differentiation (Fazi et al., 2005), and during osteoclastic differentiation (Sugatani and Hruska, 2009). Ablation of *MEF2C*, which modulates cell-fate decision of HSCs in mammals (Schüler et al., 2008; Stehling-Sun et al., 2009), suppresses proliferation of granulocyte progenitors, correcting the mice phenotype promoted by miR-223 knockout (Johnnidis et al., 2008), while ablation of miR-223 was shown to

increase proliferation of eosinophil progenitors through an up-regulation of *IGF1R* (Lu et al., 2013). Both *MEF2C* and *IGF1R* genes were validated as miR-223 targets (Jia et al., 2011; Johnnidis et al., 2008). Although *NFIA*, *MEF2C* and *IGF1R* were not linked to zebrafish hematopoiesis so far, a possibility which should be further investigated in detail, previous studies indicate that several other functions attributed to those genes are conserved in zebrafish (Hinits and Hughes, 2007; Hinits et al., 2012; Pistocchi et al., 2013; Schlueter et al., 2007) and thus it is possible that their function in hematopoiesis could also be conserved.

Another process that has been linked to miR-223 activity is tumorigenesis, including malignant hematopoiesis. In cancer, miR-223 is commonly deregulated either being silenced or over-expressed (Haneklaus et al., 2013; Vasilatou et al., 2010) and, it targets important cell cycle regulators such as *E2F1*, *STMN1*, *FOXO1*, *FBXW7* (Kang et al., 2012; Kurashige et al., 2012; Mansour et al., 2013; Pulikkan et al., 2010; Wong et al., 2008; Wu et al., 2012), suggesting that miR-223 might function as an oncomiR or a tumor suppressor miRNA. Since transgenic mutant zebrafish lines are already available, either modelling lymphoblastic leukemia (ALL) or myeloid leukemia and myeloproliferative disorder (AML/MDS) (Shen et al., 2013; Teittinen et al., 2012), these could be useful tools to help unveil the regulatory mechanisms of miR-223 in leukemia.

Finally, miR-223 also seems to play an important role in vascular development. Accordingly, miR-223 is expressed in vascular smooth muscle cells (VSMCs), and its overexpression was shown to increase proliferation and migration, probably by targeting *MEF2C* and ras homolog family member B (*RHOB*) (Rangrez et al., 2012). From the pool of genes that were previously validated as miR-223 targets in other processes, several have functions associated to angiogenesis and cardiovascular development, both in mammals and zebrafish including *RASA1* (Kranenburg et al., 2004; Ren et al., 2013), *LMO2* (Yamada et al., 2000; Zhu et al., 2005), *FBXW7* (Izumi et al., 2012), *MEF2C* (Edmondson et al., 1994; Hinits et al., 2012) and *IGF1R* (Galer et al., 2011; Huang et al., 2013). Although the role of miR-223 in those processes is

still uncharacterized, the number of known targets with established roles in one of the mentioned processes suggests that this miRNA could have a physiological or pathological role in at least one of them. Once more, zebrafish could be a valuable model to investigate the possible involvement of miR-223 in those pathways. In that sense, several studies have already clearly demonstrated the suitability of zebrafish to investigate miRNAs in vascular and heart development (Gays and Santoro, 2013).

2.6. Conclusions

Our data revealed that miR-223 structural and functional features have been conserved throughout evolution. Its genomic organization and context are maintained between human and zebrafish, and conservation of primary and secondary structures of miR-223 precursors suggests that processing and function of miR-223 might be maintained across vertebrates. We provide additional evidence supporting the use of zebrafish as model to study miR-223 function by showing that its expression pattern during development is consistent with a role in primitive and definitive hematopoiesis and by predicting as putative targets of zebrafish miR-223 genes with mammalian orthologs already shown to be involved in hematopoiesis. The data presented here contributes decisively to define a pool of miR-223 target genes and physiological processes including hematopoiesis, osteoclastogenesis, malignancy and cardiovascular development, that should be further investigated in order to clarify the role of miR-223. Furthermore, we provide evidence supporting the, use of zebrafish as model to study those putative functions.

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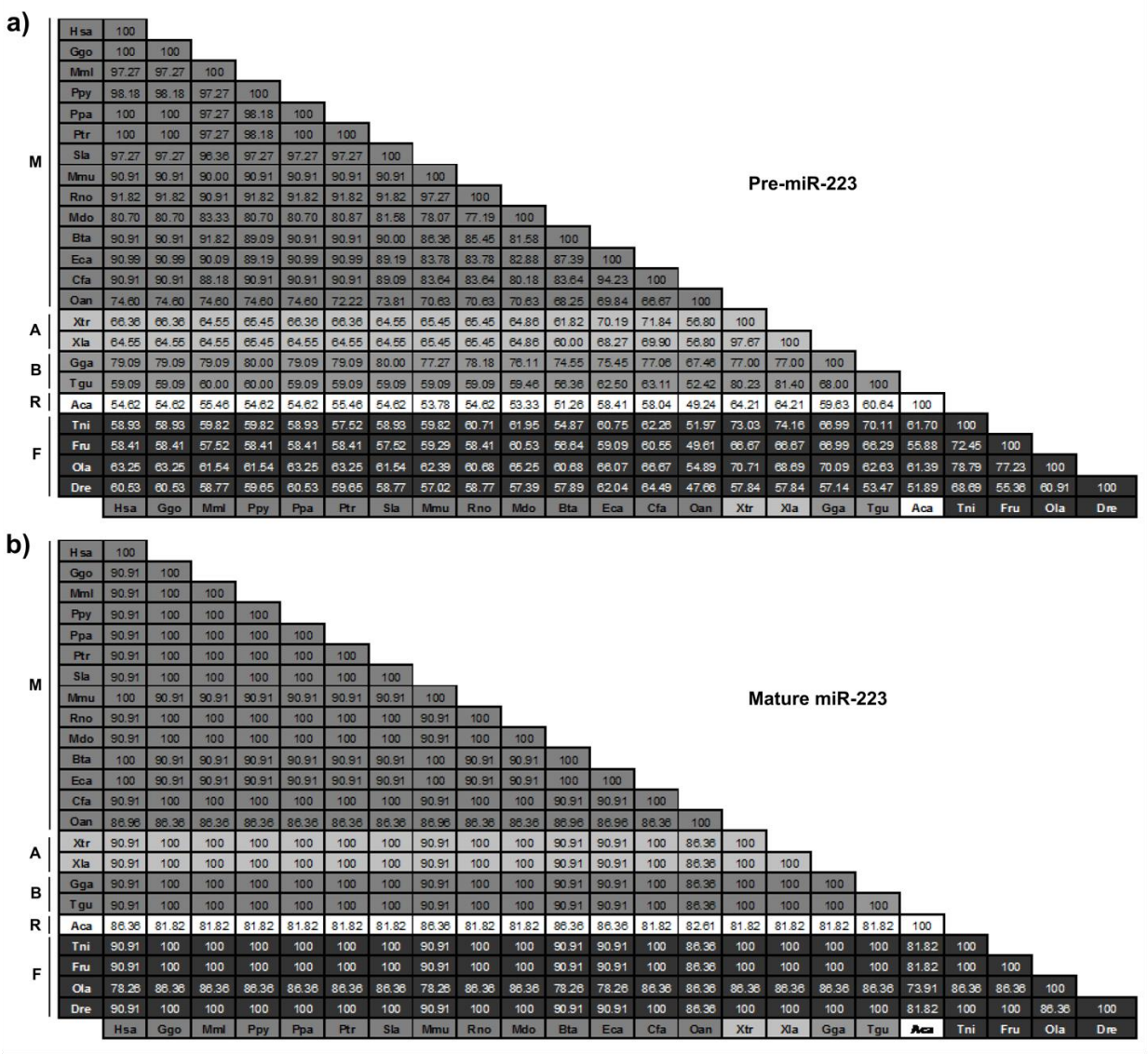
2.7. Supplementary Material

Name	Sequence
Primers used for cloning of pri-miR-223	
Dre pri-miR-223 Fw1	5'-AGGTCCATCAGCACACCCCGTCTC-3´
Dre pri-miR-223 Fw2	5'-CAGACTGTGGTTGACACTCGATCTAAAGG-3'
Dre pri-miR-223 Rev1	5'-GAGAGAGTAACAGGATCGCATGGGTCA-3'
Dre pri-miR-223 Rev2	5'-GAATTCTGAAGACTCGTGCCCCCTC-3'
qPCR primers	
Mmu miR-223 Fw	5´-TGTCAGTTTGTCAAATACCCC-3'
Mmu U6 Fw	5´-AGGATGACACGCAAATTCGTG-3´
Dre miR-223 Fw	5´-TGTCAGTTTGTCAAATACCCC-3'
Dre U6 Fw	5'-AGGATGACACGCAAATCCGTG-3'
Comercial primers	
oligo-d(T)-adapter primer	5'-ACGCGTCGACCTCGAGATCGATG(T)13 - 3'
AP1 (Marathon library specic primer)	5´-CCATCCTAATACGACTCACTATAGGGC-3'
AP2 (Marathon library specic primer)	5´-ACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3'

Supplementary Table 2.1. List of primers used in this study.



Supplementary Figure 2.1. Pairwise percent identities of pre-miR-223 (a) and mature miR-223 **(b)** sequences in different vertebrate species. Different groups of organism are indicated by shaded areas with corresponding letters: M - mammals, A - amphibians, B- birds, R - reptiles and F - fish. The identity values were calculated from alignments using Clustal Omega. Sequences were obtained from the following species: *Anolis carolinensis* (Aca), *Bos taurus* (Bta), *Canis familiaris* (Cfa), *Danio rerio* (Dre), *Equus caballus* (Eca), *Gallus gallus* (Gga), *Gorilla gorilla* (Ggo), *Homo sapiens* (Hsa), *Macaca mulatta* (Mml), *Monodelphis domestica* (Mdo), *Mus musculus* (Mmu), *Oryzias latipes* (Ola), *Ornithorhynchus anatinus* (Oan), *Pan paniscus* (Ppa), *Pongo pygmaeus* (Ppy), *Pan troglodytes* (Ptr), *Rattus norvegicus* (Rno), *Saguinus labiatus* (Sla), *Taeniopygia guttata* (Tgu), *Takifugu rubripes* (Fru), *Tetraodon nigroviridis* (Tni), *Xenopus laevis* (Xla) and *Xenopus tropicalis* (Xtr).

CHAPTER 3

miR-29a is an enhancer of mineral deposition in bone-derived systems

CHAPTER 3 • miR-29a is an enhancer of mineral deposition in bone-derived systems

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3.1. Abstract

MicroRNAs (miRNAs) provide a mechanism for fine-tuning of intricate cellular processes through post-transcriptional regulation. Emerging evidences indicate that miRNAs play key roles in the regulation of osteogenic differentiation and bone formation. The miR-29 family was previously implicated in osteoblast differentiation of mammals by targeting extracellular matrix molecules and modulating Wnt signalling through a feedback loop. Nevertheless, the function of miR-29 in bone formation and homeostasis is not completely understood. Here, we provide novel insights into the biological effect of miR-29a overexpression in a cell model capable of *in vitro* mineralization (ABSa15 cells). The phenotype obtained from miR-29a gain of function experiments was a significant increase of extracellular matrix mineralization, probably due to accelerated differentiation. We also demonstrated for the first time that miR-29a promotes an induction of β-catenin protein levels, implying a stimulation of canonical Wnt signalling. It was further shown that SPARC is a conserved putative target of miR-29, and thus may contribute to the phenotype observed in ABSa15 cells. Finally, we provide evidences for miR-29 conservation throughout evolution based on sequence homology, synteny analysis and expression patterns. Concluding, miR-29a is a key player in osteogenic differentiation, leading to increased mineralization *in vitro*, and this

function seems to be conserved throughout vertebrate evolution by interaction with canonical Wnt signalling and conservation of targets.

3.2. Introduction

Skeletogenesis is a tightly regulated process orchestrated by numerous molecular determinants and cellular activities (Karsenty, 2008; Karsenty and Wagner, 2002). Although this process has been greatly investigated, its post-transcriptional regulators are generally unknown. MicroRNAs (miRNAs), an abundant class of small noncoding RNAs, provide a mechanism for fine-tuning of complex cellular processes through binding to the 3'-untranslated region (3'-UTR) of mRNA transcripts to attenuate protein synthesis (Tétreault and De Guire, 2013). Known to control numerous biological processes (Guarnieri and DiLeone, 2008), miRNAs were recently implicated in skeletogenesis and, in combination with key transcription factors and signalling molecules, help to control the complex program of bone formation (Zhao et al., 2013). This was evidenced by studies where conditional deletion of Dicer, an enzyme crucial for miRNA biogenesis, in osteoprogenitor cells and in chondrocytes resulted in abnormal formation of bone and cartilage of mouse (Gaur et al., 2010; Kobayashi et al., 2008). Other studies have identified a panel of miRNAs that act as negative regulators of bone formation. For instance, miR-206 targeting of connexin43 (Cx43) was shown to impede osteoblast differentiation both *in vitro* and *in vivo* (Inose et al., 2009). A set of 11 miRNAs was found to target the master regulator of osteogenesis, RUNX2, in both osteoblasts and chondrocytes, and to inhibit osteoblast differentiation (Zhang et al., 2011c). Furthermore, miR-214 was shown to repress ATF4, a transcription factor that orchestrates osteoblast differentiation and function, which consequently inhibited osteoblast activity and matrix mineralization *in vitro* and bone formation *in vivo* (Wang et al., 2013a). The opposite effect of miRNAs in bone, i.e. positive regulation of osteoblast differentiation, was also demonstrated. For instance, miR-2861 overexpression enhanced osteoblastogenesis in mouse bone marrow stromal cells, whereas its silencing *in vivo* decreased Runx2 protein levels and inhibited bone formation, by targeting HDAC5 (Li et al., 2009a). Zhang and co-

workers demonstrated that miR-335-5p down-regulates DKK1, thus activating Wnt signalling and promoting osteogenic differentiation (Zhang et al., 2011b). Among this group of miRNAs is the miR-29 family, which expression was shown to increase during mouse osteoblast differentiation (Kapinas et al., 2009; Li et al., 2009b). This family is composed by miR-29a, miR-29b and miR-29c, which share the same seed sequence and collectively target several genes associated to extracellular matrix (ECM) in bone, including several collagens, matrix metalloproteinase 2 (MMP2), MMP9 and SPARC (secreted protein acidic and rich in cysteine) (Kapinas et al., 2009; Li et al., 2009b; Rossi et al., 2013). In this regard, miR-29b was also found to target known inhibitors of osteoblast differentiation, i.e. HDAC4, TGF3, ACVR2A, CTNNBIP1, and DUSP2 (Li et al., 2009b), and to promote osteogenesis in mouse. More recently, miR-29b was shown to target osteo-inhibitory genes CDK6, HDAC4 and CTNNBIP1, in human somatic stem cells and to accelerate osteogenic differentiation (Trompeter et al., 2013). MiR-29a was suggested to positively regulate osteoblast differentiation by repression of SPARC, an important protein for ECM assembly and deposition (Kapinas et al., 2009). Furthermore, in mammalian osteoblasts, the transcription of miR-29a was shown to be induced by a key pathway of bone formation, the Wnt signalling, and in turn, miR-29a was shown to repress three antagonists of Wnt, thus potentiating its signalling cascade and contributing for differentiation (Kapinas et al., 2010). Despite all evidences concerning miR-29 effect on mammalian osteogenic differentiation, characterization of its function and regulatory mechanisms in other organisms is far from being understood, which could help to elucidate the intricate and extensive role of this miRNA family. In this regard, teleost fish not only present several anatomic, physiologic and genetic similarities with mammals, but also present several experimental advantages, such as transparent larvae (crucial for developmental characterization of systems), large progeny or easy manipulation (transgenic preparation), which make them suitable models to investigate vertebrate development, including skeletogenesis (Spoorendonk et al., 2010). In fact, several miRNAs have been investigated in fish models, both *in vivo* and *in vitro* (He et al., 2011; Tiago et al., 2014) and contribute to

elucidate their roles in skeletal formation, demonstrating the suitability of these models.

In this work, we investigated the biological effects of miR-29a overexpression in a fish bone-derived cell line, the ABSa15, capable of *in vitro* mineralization and suitable for miRNA studies (Marques et al., 2007; Tiago et al., 2014). We bring novel data regarding miR-29a effect on *in vitro* mineral deposition and osteogenic differentiation as determined by specific marker genes. We also provide information regarding conservation of miR-29a mechanisms of action and regulation in vertebrates.

3.3. Materials and Methods

3.3.1. Cell culture maintenance

The ABSa15 cell line was recently deposited in the European Collection of Cell Cultures (Ref. 13112201). This cell line was previously developed in our laboratory from calcified branchial arches of the gilthead seabream (*Sparus aurata*, Linnaeus, 1758) and is capable of *in vitro* mineralization (Marques et al., 2007). Also, ABSa15 cells express several genes known as markers for differentiation and mineralization (Tiago et al., 2014). ABSa15 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (SIGMA), 1% penicillin/streptomycin, 0,2% Fungizone and 2 mM L-Glutamine (all from GIBCO BRL, Gaithersburg, MD) at 33ºC in 10% CO_2. Cells were sub-cultured every 2-3 days through trypsinization.

3.3.2. Extracellular matrix (ECM) mineralization

ASBa15 cells were seeded at a density of 2 x 10^4 cells/ well in 24-well plates. ECM mineralization was induced in confluent cultures through the addition of L-ascorbic acid (50 µg/ml), β-glycerophosphate (10 mM) and $CaCl_2$ (4 mM) to the growth medium. Differentiation medium was renewed every 3-4 days. At appropriate times, mineral deposition was revealed through von Kossa staining and quantified by densitometry analysis or through alizarin red S

staining and spectrophotometric quantification as described elsewhere (Pombinho et al., 2004; Stanford et al., 1995; Tiago et al., 2014).

3.3.3. RNA extraction and quantitative real-time PCR (qPCR) analysis

Total RNA was extracted from confluent cultures, at appropriate times, as previously described (Chomczynski and Sacchi, 1987) and quantified by UV spectrophotometry (NanoDrop ND-1000). Quantitative real-time PCR (qPCR) analysis of miRNAs and mRNAs was performed using the StepOnePlus system (Applied Biosystems). For qPCR analysis of miRNAs, total RNA (1 µg) was treated with RQ1 RNase-free DNase (Promega), then polyadenylated, reverse-transcribed and amplified using miRNA-specific primers (Supplementary Table 1) and the NCode miRNA First-Strand cDNA Synthesis and NCode SYBR miRNA qRT-PCR kits (Invitrogen), according to manufacturer's instructions. For qPCR analysis of mRNAs, 1 µg of total RNA was treated with RQ1 RNase-free DNase, then reverse-transcribed using MMLV-RT (Invitrogen) and oligo-d(T)-adapter primer (Sup. Table 1), according to manufacturer's instructions. For analysis of miRNAs, PCR amplifications were performed using 1.6 ng of cDNA, gene-specific primers (Sup. Table 1) and Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), according to manufacturer's instructions. For analysis of mRNAs, PCR amplifications were performed using 10 ng of cDNA, gene-specific primers (Sup. Table 1) and SsoFast EvaGreen Supermix (Bio-Rad), according to manufacturer's instructions. Relative mRNA and miRNA expression was calculated using the ΔΔCt method (Livak and Schmittgen, 2001) and normalized using expression levels of ribosomal protein L27a (RPL27a) and U6 small nuclear RNA (U6), respectively.

3.3.4. Establishment of stable fish cell clones overexpressing miR-29a

For miR-29a overexpression, oligonucleotides containing forward and reverse sequences of zebrafish pre-miR-29a were annealed and inserted into pcDNA6.2-GW/EmGFP-miR vector downstream of GFP coding sequence using the BLOCK-iT Pol II miR RNAi Expression Vector kit (Invitrogen), according to

manufacturer's instructions. Identity and integrity of inserted fragments were confirmed through sequencing.

ABSa15 cells were seeded in 6-well plates at $2x10^5$ cells/well, cultured for 14-16 hours and transfected with 2.4 µg of pcDNA6.2/EmGFP-miR29a construct and FugeneHD (Roche), according to manufacturer's instructions. After 24 h, cells were sub-cultured into a 10-cm culture dish and blasticidin selection antibiotic was added to cell culture medium (optimal concentration of 2 µg/ml of medium). The amount of antibiotic to be used was determined as described in the manual of BLOCKiT Pol II miR RNAi Expression Vector kit. After 30 days in selective medium (renewed twice a week), cell colonies expressing GFP were identified using Olympus IX-81 fluorescence microscope, and then sequentially sub-cultured into 48-well, 24-well, 6-well and 10-cm culture dishes. Positive clones were assessed for miR-29a expression by qPCR analysis and further characterized for ECM mineralization, as described above.

3.3.5. Transient overexpression of miR-29a during ECM mineralization

miRIDIAN microRNA mimic for dre-miR-29a (denominated MmiR-29 from now on) or negative control 1 (NC) (both obtained from Dharmacon) were delivered in triplicates to ASBa15 cells at a final concentration of 50 nM using EzWay (Koma Biotech) transfection reagent, according to manufacturer's instructions. ASBa15 cells were seeded at a density of 2×10^4 cells/ well in 24-well plates and transfected 16 hours later. When cells reached confluence (3 days after), mineralization cocktail (described above) was added to medium (T0). A second transfection was performed after 14 days (T14), using the same procedure. After 28 days (T28), ECM mineralization was revealed and quantified through alizarin red S staining, as described above. In parallel, total RNA was extracted from transfected cells and further analysed by qPCR.

3.3.6. Vector constructions

For luciferase assays, the 3'- UTR of gilthead seabream *SPARC* (also known as osteonectin) transcript was inserted into *XbaI* site of pGL3-Control vector (Promega) downstream of firefly luciferase (F-Luc) coding sequence.

3'UTR was amplified from Marathon cDNA libraries (Clontech) using gene-specific primers (listed in Table 1) and Klen Taq Polymerase mix (Clontech). p-CT, p-SPARC-S and p-SPARC-AS will be used from this point forward to denominate the pGL3-Control vector empty or containing either the sense or antisense SPARC 3´UTR, respectively.

3.3.7. Dual-Luciferase Reporter Assays

ABSa15 cells were seeded in 12-well plates at 8×10^4 cells/well, further cultured for 14–16 hours and transfected with 600 ng of p-CT, p-SPARC-S or p-SPARC-AS and 600 ng of pRL-SV40 vector (Promega) using FuGene HD (Roche), according to manufacturer's instructions. After 48 hours, cells were lysed in appropriate buffer and luciferase activities measured using Dual-Luciferase Reporter Assay system (Promega), according to manufacturer's instructions. Relative luciferase activity was determined from the ratio F-Luc/R-Luc.

3.3.8. Western Blot analysis

Cell extracts were prepared in lysis buffer containing Tris (50 mM), sodium chloride (150 mM), NP-40 (1% m/v), glycerol (10% v/v), magnesium chloride (10 mM), sodium orthovanadate (10mM) and protease inhibitor cocktail (cOmplete, Roche). After centrifugation (15,000 g, 15 min, 4ºC), pellet was discarded and protein content was determined in the supernatant using the Bradford reagent (Bio-Rad), according to manufacturer's instructions. Proteins were fractioned by electrophoresis using NuPAGE Novex Bis-Tris Gels (Invitrogen; 4-12% acrylamide) and transferred onto PVDF 0.45 μm membranes (Millipore) using the XCell SureLock blot module (Invitrogen). The following antibodies were used for identification of respective proteins: anti-human β-Catenin rabbit IgG conjugate (Santa Cruz Bio- technology; 1:500 dilution), anti-avian b-Actin mouse IgG conjugate (Santa Cruz Bio- technology; 1:500 dilution), anti-rabbit IgG peroxidase conjugate (Sigma–Aldrich; 1:30,000 dilution) and anti-mouse IgG-peroxidase conjugate (Sigma–Aldrich; 1:30,000 dilution). Protein – antibody peroxidase conjugates were detected using the

Western Lightning ECL kit (Perkin Elmer). Chemiluminescent signal was detected using Hyperfilm ECL (Amersham, GE Healthcare) and quantified by densitometry analysis.

3.3.9. Statistical analysis

Statistical analysis was performed with GraphPad Prism 5 (GraphPad, La Jolla, CA). Comparisons between two groups were made using a two-tailed Student's t-test. For comparisons between multiple groups, one-way ANOVA followed by Newman-Keuls's HSD post-hoc test, was used. Differences were considered statistically significant for $p<0.05$.

3.4. Results

3.4.1. miR-29 family conservation in vertebrates

The miR-29 family has three mature miRNA members, encoded in two clusters. MiR-29b is present in both clusters, preceding either miR-29a or miR-29c So, miR-29b has two different precursors, miR-29b-1 and miR-29b-2, which produce identical mature sequences called miR-29b. Exceptionally, additional variants belonging to this family can be found in some species (miR-29d - *Xenopus tropicalis*, *Bos taurus*, *Petromyzon marinus* - and miR-29e - *B. taurus*). Although mature miRNAs of miR-29 family were shown to be highly conserved between human, mouse and rat, so far this feature was not analysed in other vertebrate species. Thus, in order to further characterize miR-29 family conservation among vertebrates, mature miR-29 sequences (available in miRbase database release 20; http://www.mirbase.org) were collected and aligned using Clustal Omega tool (http://www.ebi.ac.uk/Tools/msa/clustalo/). According to this analysis, miR-29a and miR-29c showed higher homology, being aligned together, whereas miR-29b was somewhat different due to specific features observed in the 3' end regions (Supp. Fig. 3.1). It was concluded that miR-29 family is remarkably conserved among vertebrates and that the seed region (nucleotides 2-8), a feature with a key role in target interaction, is 100% identical for the three miRNAs in 28 analysed species.

Interestingly, it was noted that mammalian miR-29a is different from other species in one middle position. Therefore, although miR-29a is present in other non-mammalian taxa, its sequence is identical to mammalian miR-29c (Sup. Fig. 3.1). This fact raised the hypothesis that non-mammalian miR-29a could be an ortholog of mammalian miR-29c. To test this hypothesis, a gene synteny analysis of both clusters (miR-29b/a and miR-29b/c) was performed in six species belonging to five different taxa (mammalian, reptiles, birds, amphibian and teleosts) (Fig. 3.1). In humans, the gene encoding the cluster miR-29b-1/a is located in chromosome (Chr) 7, and syntenic genes identified upstream this location included potassium voltage-gated channel, Shal-related family, member 2 (*KCND2*), tetraspanin 12 (*TSPAN12*), inhibitor of growth family, member 3 (*ING3*), cadherin-like and PC-esterase domain containing 1 *(CPED1*) and wingless-type MMTV integration site family, member 16 (*WNT16*). Conversely, muskelin 1, intracellular mediator containing kelch motifs (*MKLN1*), plexin A4 (*PLXNA4*), coiled-coil-helix-coiled-coil-helix domain containing 3 (*CHCHD3*), exocyst complex component 4 (*EXOC4*) and leucine-rich repeats and guanylate kinase domain containing (*LRGUK*) were shown to be present downstream this location (Fig. 3.1a). The order and orientation of this gene arrangement was fully preserved in zebrafish Chr 4, the chromosomic location of the miR-29b-2/a cluster. In chicken, xenopus and medaka, the orientation of this miR-29 cluster is reversed as well as some syntenic genes. All syntenic genes were present in Carolina anole but located downstream this cluster. Their orientations were preserved in comparison to human and zebrafish. All clusters analysed in this group included miR-29a, suggesting that miR-29a of non-mammalian species is a true ortholog of mammalian miR-29a (Fig. 3.1a). Regarding miR-29b/c cluster, syntenic genes were only found downstream of miR-29 genomic location in human Chr 1: CTTNBP2 N-terminal like (*CTTNBP2NL*), wingless related MMTV integration site 2b (*WNT2B*), capping protein (actin filament) muscle Z-line, alpha 1 (*CAPZA1*), ras homolog family member C RHOC, solute carrier family 16 (monocarboxylate transporter), member 1 (*SLC16A1*), synaptonemal complex protein 1 (*SYCP1*), thyroid stimulating hormone, beta TSHB, nerve growth factor (beta polypeptide) (*NGF*),

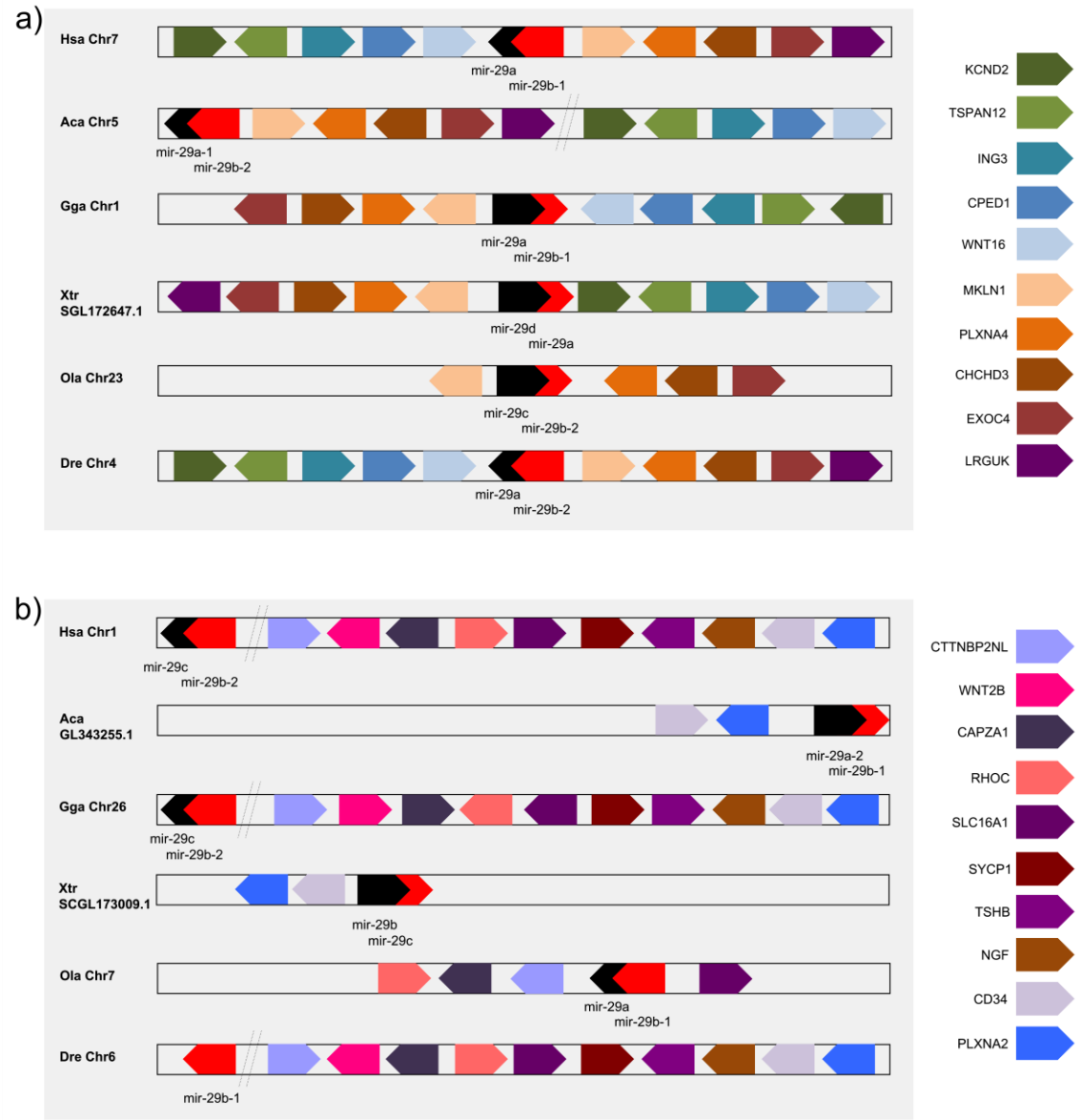

Figure 3.1. Gene synteny analysis in vertebrates relative to the host genes encoding (a) miR-29a/b and (b) miR-29c/b clusters. Representation of the genomic regions flanking vertebrate miR-29 clusters were obtained using data from the Ensembl project and Synteny database (http://syntenydb.uoregon.edu/synteny_db/). The physical localization and orientation of the genes present in the vicinity of human miR-29 clusters is indicated for mammalian, reptile, sauropsidian, amphibian and teleost fish species. Taxa are labelled as: Hsa (*Homo sapiens*), Aca (*Anolis carolinensis*), Gga (*Gallus gallus),* Xtr (*Xenopus tropicalis*), Ola (*Oryzias latipes*), Dre (*Danio rerio*). SCGL in *Xenopus tropicalis* and GL in *Anolis carolinensis* represent sequences that have not been allocated to a specific chromosome. Chr - chromosome.

CD34 molecule (*CD34*), plexin A2 (*PLXNA2*) (Fig. 3.1b). Again, from all analysed species, only zebrafish showed a complete conservation in the order and orientation of syntenic genes when compared to human. In zebrafish, this

cluster was located in Chr 6 and contained only one miRNA, miR-29b-1. In chicken, miR-29b-2/c cluster is located in Chr 26 and although all ten syntenic genes were present downstream this location and gene order was preserved, gene orientation was different in half of these genes. The other three species analysed showed the lowest synteny conservation, being identified only 2-4 syntenic genes (Fig. 3.1b). In general, this analysis strongly suggested that in non-mammalian species, miR-29a is a true ortholog of mammalian miR-29a. Furthermore, this analysis revealed a remarkable genomic conservation, in both order and orientation, between human and zebrafish chromosomes.

3.4.2. Zebrafish and mouse have similar miR-29 expression patterns

To further elucidate the putative conservation between non-mammalian and mammalian miR-29a, the expression patterns of this miRNA were investigated in zebrafish and mouse adult tissues. Thus, miR-29a expression was analysed by qPCR in zebrafish and mouse soft tissues, i.e. brain, heart and muscle, in zebrafish calcified tissues, i.e., branchial arches, vertebrae and skull, and in mouse calcified tissues, i.e. ear, femur, vertebrae and calvaria (Fig. 3.2). In zebrafish, miR-29a was detected in all analysed tissues although its expression was higher in brain (over 2-folds comparing to other tissues) (Fig. 3.2a). Like in zebrafish, mouse miR-29a was also detected in all analysed tissues and though its levels of expression were high in the brain, the highest expression was found in heart (3-fold change over the brain) (Fig. 3.2b). In mouse, the muscle had similar levels of expression as in brain, whereas in calcified tissues miR-29a relative expression was considerably lower (Fig. 3.2b). Mouse miR-29c expression pattern resembled the one observed for miR-29a (Sup. Fig. 3.2), in agreement with previous results (Kapinas et al., 2009). These data indicate that miR-29a has a similar pattern of expression in zebrafish and mouse, and further confirm that, in mouse, both miR-29a and miR-29c share the same sites of expression.

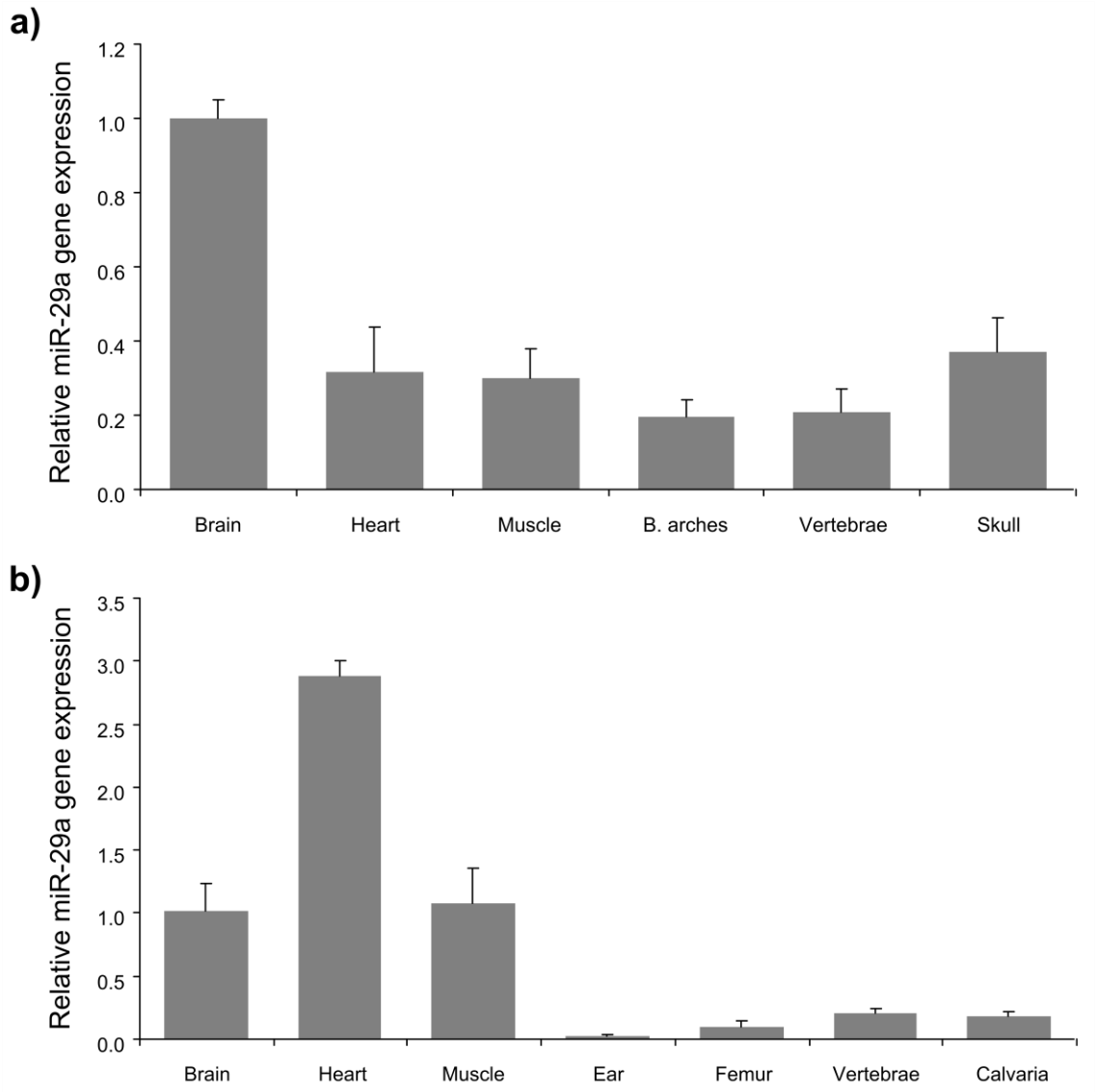

Figure 3.2. Relative miR-29a expression in (a) zebrafish and (b) mouse adult tissues. Levels of miR-29a expression were measured by miRNA specific qPCR analysis, using total RNA samples of zebrafish and mouse adult tissues, and normalized using U6 small RNA and brain as reference sample. Values are the mean of at least 3 independent replicates. B. arches – Branchial arches.

3.4.3. miR-29a is up-regulated during ECM mineralization of ABSa15 cells

Since in mammals miR-29 was previously shown to promote osteoblast differentiation through a variety of mechanisms (Kapinas et al., 2009, 2010; Li et al., 2009b), and here it was shown that (i) miR-29 is highly conserved between fish and mammals, and (ii) zebrafish and mouse share similar patterns of expression, we asked whether miR-29 could promote similar effects in fish. To

address this question we used ABSa15 cells, a cell line derived from calcified branchial arches of the gilthead seabream, that is capable of *in vitro* mineralization (Marques et al., 2007; Tiago et al., 2014). Furthermore, this cell line was recently proven to be a suitable cell line to investigate miRNA effects on fish osteogenic differentiation and mineralization (Tiago et al., 2014). First, the pattern of expression of miR-29a was characterized during ABSa15 cell differentiation, i.e. at confluence (day 0), and after 7, 14, 21 and 28 days of treatment with mineralogenic medium (Fig. 3.3). According to qPCR analysis, the expression of mature miR-29a was strongly increased during differentiation of ABSA15 cells, approximately a 10-fold change comparing 0 and 7 days (Fig. 3.3). These levels of expression were maintained high during ECM mineralization, which occurred between days 14 and 28 (as determined by von Kossa staining, data not shown). This expression pattern suggested that miR-29a is required to support ABSa15 cell differentiation and ECM mineralization.

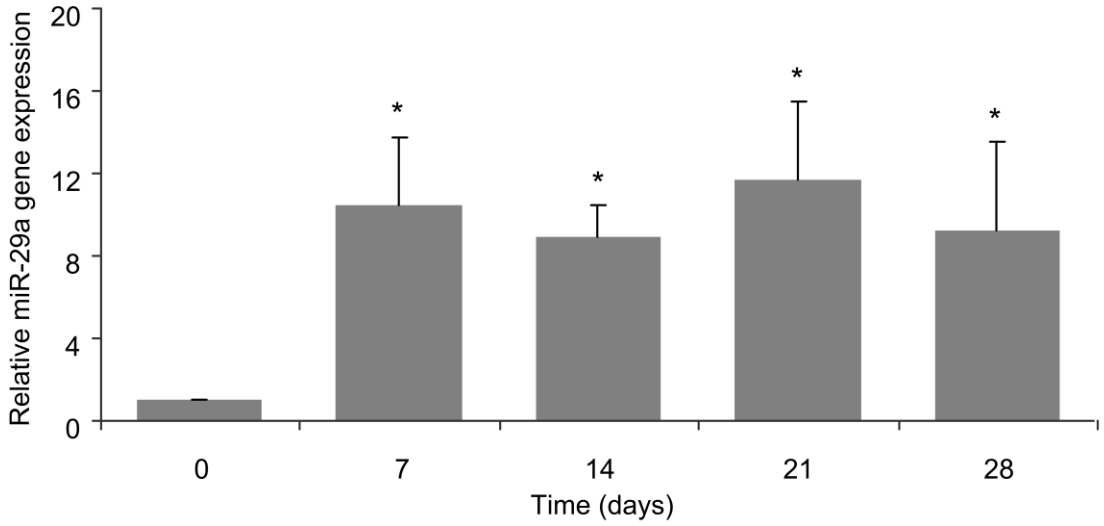

Figure 3.3. Relative miR-29a expression during ABSa15 cell differentiation. Levels of miR-29a expression were determined by qPCR analysis, using total RNA samples collected from differentiating ABSa15 cells and normalized using U6 small RNA expression and control time 0 (C0) as reference. Asterisks (*) indicate values statistically different from C0 at specific time of differentiation. Values are the mean of at least 3 independent replicates. (Student's t-test, $p < 0.001$).

3.4.4. miR-29a promotes ECM mineralization in ABSa15 cells

To study the functional activity of miR-29a during ECM mineralization, clones of ABSa15 cells overexpressing miR-29a were generated through stable transfection of pcDNA6.2-GW/EmGFP-miR-29a constructs. Although seven clones homogeneously expressing GFP (data not shown) were obtained, only one clone was confirmed to overexpress miR-29a by qPCR with a ~ 3-fold increase over wild type (WT) cells (Fig. 3.4a). Generation of stable cell lines overexpressing miR-29 was previously attempted by other authors, without success, and it was suggested that miR-29 could enforce a strong negative selection against cell growth *in vitro* (Chang et al., 2008; Kapinas et al., 2009). The obtained clone (OE-miR-29a) was then cultured in mineralogenic medium for 28 days and mineral deposition evaluated once a week through von Kossa staining. ECM mineralization was evident in WT cells after 21 days of treatment and, at that time, mineral deposition was significantly increased in OE-miR-29 cells by 4-fold (Fig. 3.4b, c). By day 28, mineral deposition in WT and OE-miR-29a cells were still significantly different, although to a lower extent (Fig. 3.4b, c). These findings suggested that miR-29a is either able to induce ECM mineralization or to accelerate differentiation of fish bone-derived cells. In order to further confirm this result, and since we could obtain only one stable clone overexpressing miR-29a, miR-29a was transiently delivered into ABSa15 cells, and its effects investigated during ECM mineralization. Thus, ABSa15 cells were transfected with either miR-29a mimic or negative control and, at confluence, cells were exposed to the mineralogenic cocktail for 28 days. To ensure the delivery and activity of miR-29a mimic during the mineralogenic period, expression levels of miR-29a were determined by qPCR after 28 days of mineralogenic treatment. Transfections with miR-29a mimic resulted in an average increase of mature miR-29a expression of 2.5-fold change over the control (Fig. 3.5a). Regarding ECM mineralization, transient overexpression of miR-29a promoted a significant increase of approximately 70% of mineral deposition over the control, as revealed by alizarin red S staining (Fig. 3.5b, c). This result was in agreement with the findings for OE-miR29a, i.e. significant increase of ECM mineralization.

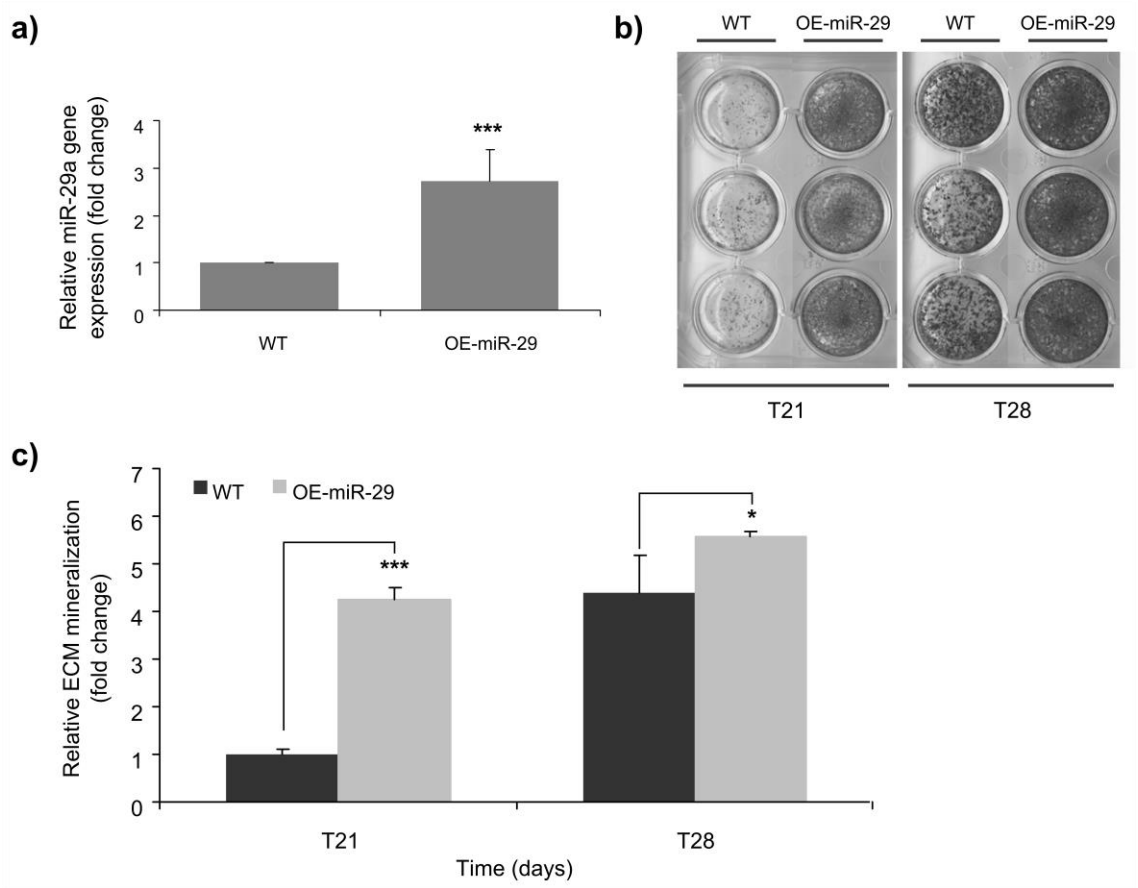

Figure 3.4. Effect of miR-29a stable overexpression in ABSa15 cells undergoing ECM mineralization. (a) Relative miR-29a expression in wild-type cells (WT) and one clone overexpressing miR-29a (OE-miR-29); levels of miR-29a expression were determined by qPCR analysis using total RNA from confluent cultures and normalized using U6 small RNA expression and WT as reference. (b) ECM mineralization in WT cells comparing to OE-miR-29; mineral deposition was revealed by von Kossa staining, after 21 and 28 days of mineralogenic treatment. (c) Densitometry analysis of ECM mineralization in WT and OE-miR-29; WT cells at 21 days was set as reference. All values in OE-miR-29 were statistically different from values in WT cells. Values are the mean of at least 3 independent replicates. (Student's t-test, *** $p < 0.001$; * $p < 0.05$).

To better understand the mechanisms underlying this effect, a set of markers of bone cell differentiation/mineralization were assessed by qPCR analysis after 28 days of mineralogenic treatment in cells transfected with miR-29a mimic and control. Among analysed genes (bone morphogenetic protein 2, *bmp2*; β-catenin, *ctnnb1*; collagen type I alpha 1, *col1a1*; matrix Gla protein, *mgp*; osteocalcin 1 and 2, *oc1* and *oc2*; secreted phosphoprotein 1, *spp1*, or also known as osteopontin, *opn*; runt related transcription factor 2, *runx2*, and tissue non-specific alkaline phosphatase from liver/bone/kidney, *tnap*, only the levels

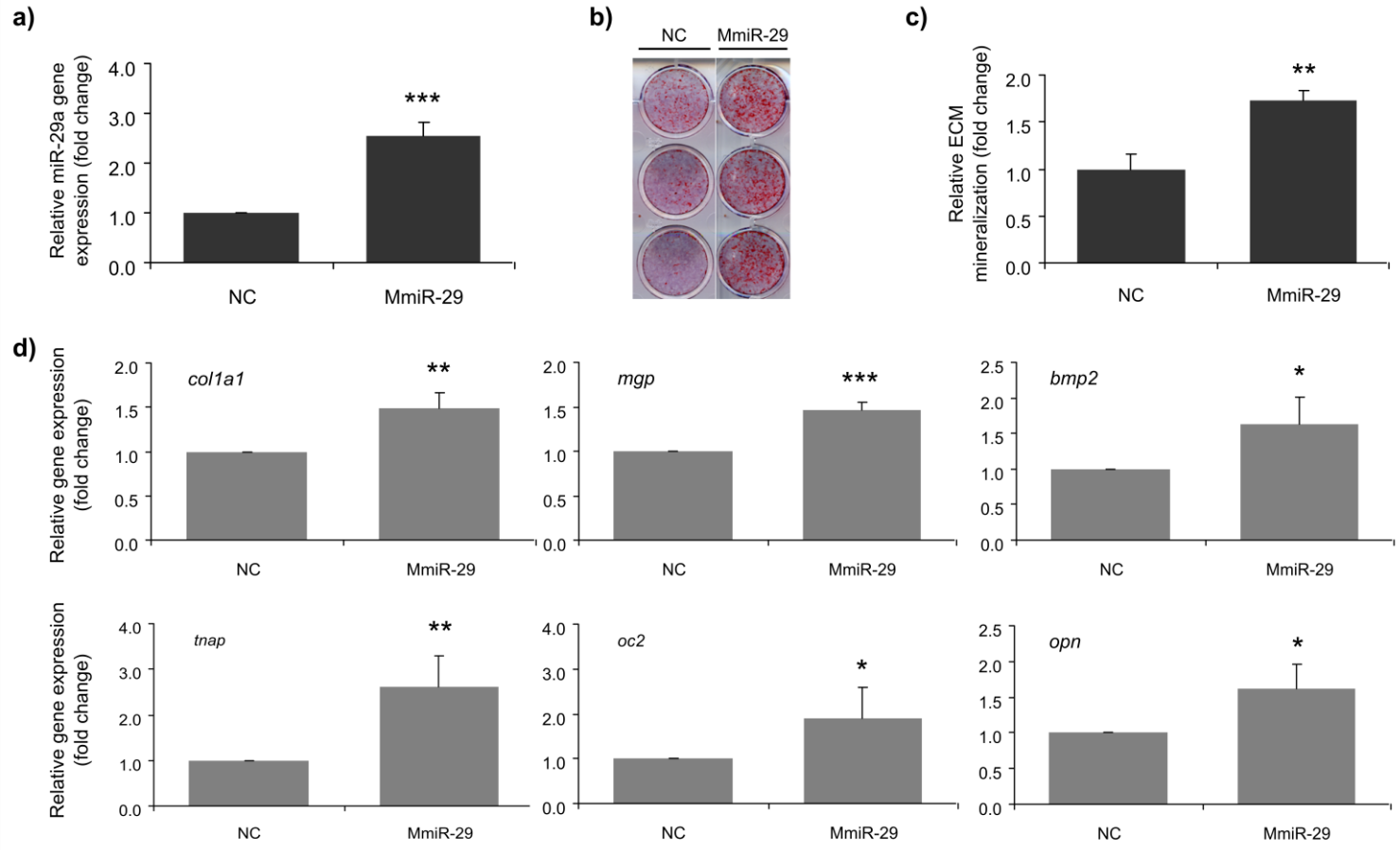

Figure 3.5. Effect of transient miR-29a overexpression in Absa15 cells undergoing ECM mineralization. Mimic of miR-29a (MmiR-29) or a negative control (NC) were transfected into ABSa15 cells and ECM mineralization was induced 3 days later (T0). After 28 days of treatment (T28), (a) expression of miR-29a, (b) ECM mineralization revealed by alizarin red S staining and (c) respective quantification of staining in both NC and MmiR-29 were assessed. Levels of miR-29a expression were determined by qPCR analysis using total RNA from T28 and normalized using U6 small RNA expression and NC as reference. (d) Relative expression of marker genes for ABSa15 cell differentiation/mineralization in MmiR-29 and NC after 28 days of mineralogenic treatment (T28): alkaline phosphatase (tnap), collagen, type I, alpha 1 (*col1a1*), matrix Gla protein (*mgp*), osteocalcin 2 (*oc2*), bone morphogenic protein 2 (*bmp2*) and osteopontin (*op*). Levels of gene expression were determined by qPCR analysis using total RNA samples from NC and MmiR-29 at T28, and normalized with RPL27a housekeeping gene expression (similar expression data was collected using 18S housekeeping gene; data not shown) and NC as reference. Asterisks indicate values statistically different from NC. Values are the mean of at least 3 independent replicates. (Student's t-test, *** $p < 0.001$; ** $p < 0.01$, * $p < 0.05$).

of expression of *col1a1*, *mgp*, *bmp2*, *tnap*, *oc2* and *opn* were shown to be significantly altered, being up-regulated (Fig. 3.5d). These data provided additional evidences for miR-29a stimulation of ABSa15 cells ECM mineralization and differentiation.

3.4.5. Seabream sparc is putatively regulated by miR-29a in ABSa15 cells

The miR-29 family was found to promote osteogenic differentiation by targeting inhibitors of osteoblastic differentiation, inhibitors of the canonical Wnt signalling and bone matrix RNAs (Kapinas et al., 2009; Li et al., 2009b; Sengupta et al., 2008). In this regard, SPARC, the most abundant non-collagen ECM protein in bone, is a known target of miR-29 during mammalian osteoblast differentiation (Kapinas et al., 2009). In order to investigate whether *sparc* is also a target of miR-29 in fish, thus contributing for the phenotype observed in miR-29 gain-of-function experiments in ABSa15 cells during ECM mineralization, we first verified the evolutionary conservation of the binding sites described by Kapinas and colleagues (Kapinas et al., 2009) using TargetScanMouse Release 6.2 (http://www.targetscan.org/mmu_61/). The two binding sites for miR-29 were highly conserved in twenty mammalian species, one reptile, one bird and one amphibian present in the database (data not shown). Since this database did not contain any *sparc* transcripts of fish origin, to overcome this issue, GenBanK sequence database (http://www.ncbi.nlm.nih.gov/) was searched for vertebrate *SPARC* transcripts. This search allowed the identification of *SPARC* sequences from seven fish, two amphibian and four bird species that were used to expand our analysis. The 3´UTR region of each collected transcript was then searched for miR-29 binding sites using PITA algorithm (http://genie.weizmann.ac.il/pubs/mir07/index.html) and RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/). The two binding sites for miR-29a predicted by both algorithms are represented in Fig. 3.6 (a and b), where 3' UTRs of all analysed species were aligned along with six mammalian species using Clustal Omega tool (http://www.ebi.ac.uk/Tools/msa/clustalo/). According to this analysis, the two previously identified binding sites for miR-29 appeared to have been highly

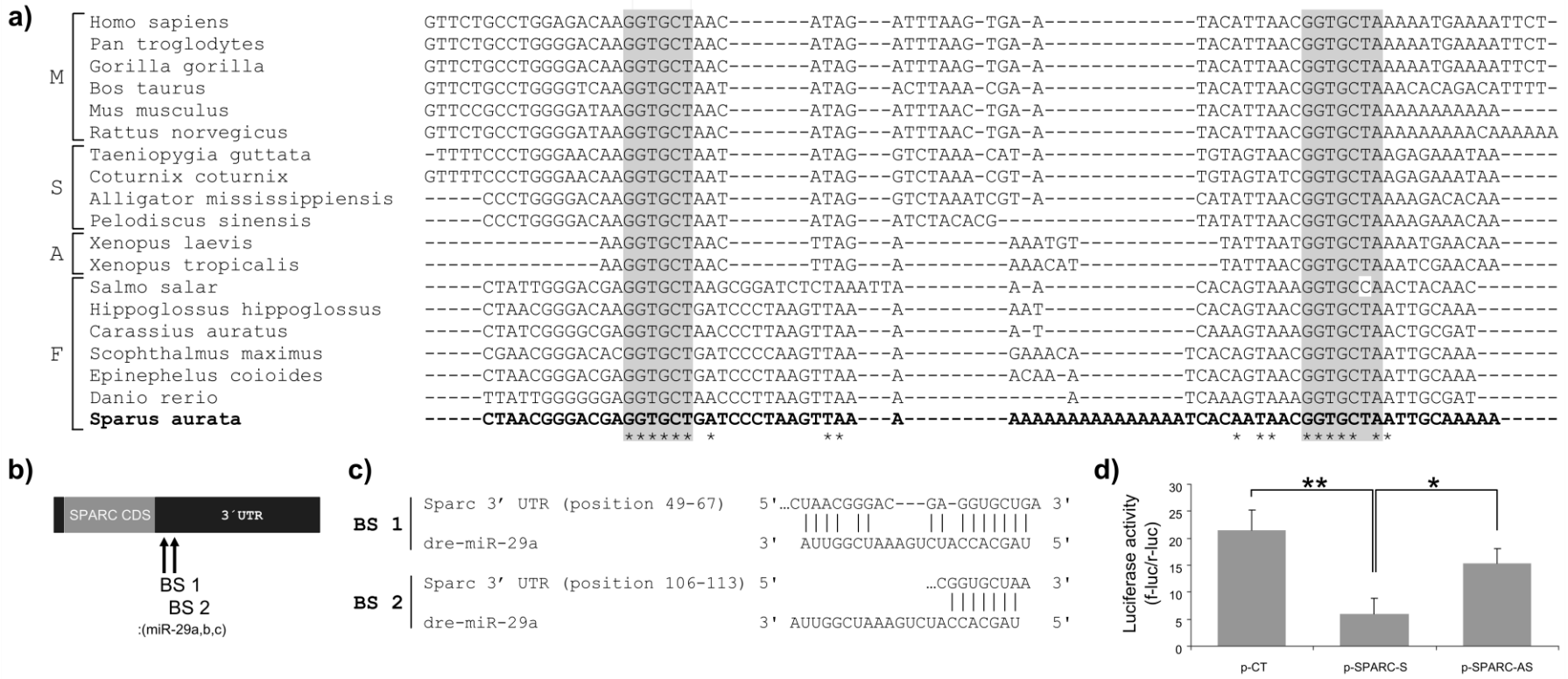

Figure 3.6. Prediction of miR-29a binding sites (BS) in the 3'UTR of gilthead seabream SPARC and respective functional characterization in ABSa15 cells. (a) Alignment of 3'UTR region of mammalian (M), sauropsids (S), amphibian (A) and fish (F) SPARC transcripts showing two consecutive and conserved miR-29 seed regions (gray box); BS of miR-29 were predicted using TargetScanHuman Release 6.2 and RNAhybrid algorithms; asterisks (*) indicate 100% conserved nucleotide positions; gilthead seabream sequence is in bold. (b) Schematic representation of consensus SPARC transcript and positions of both BS for miR-29 family. (c) Specific site within 3'UTR of gilthead seabream SPARC transcript where dre-miR-29a is predicted to bind, as determined by RNAhybrid. (d) Relative luciferase activities in ABSa15 cells transfected with constructs containing sense (p-SPARC-S) and antisense (p-SPARC-AS) sequences of 3'UTR of gilthead seabream SPARC transcript, and respective control vector (p-CT). Luciferase activity was calculated as the ratio of firefly and renilla luciferase activities. Asterisks indicate values statistically different from respective 3'UTR value. Values are the mean of at least 3 independent replicates. (one-way ANOVA, ** $p < 0.01$, * $p < 0.05$).

conserved throughout evolution and were clustered in the proximal *SPARC* 3´UTR. In seabream, the specie from which ABSa15 cells were obtained, each *sparc* binding site contained 7 nucleotides pairing to the seed of miR-29, corresponding to minimal free energies of -19.5 and -17.2 kcal/mol (Fig. 3.6c), as predicted by RNAhybrid algorithm. In order to investigate whether seabream *sparc* could be regulated by miR-29, a reporter vector containing the seabream *sparc* 3´UTR region downstream of luciferase gene was transfected into ABSa15 cells, which endogenously express miR-29a. Controls included transfection of the reporter vector alone and a construct where *sparc* 3´UTR was in the antisense orientation downstream of luciferase gene (Fig. 3.6d). The relative luciferase activity of *sparc* 3´UTR construct was significantly reduced by more than 70% over the vector alone and by approximately 60% over the construct with the *sparc* 3´UTR antisense (Fig. 3.6d). Differences between the relative luciferase activity of the vector alone and the construct containing the antisense strand of *sparc* were not statistically significant. Although other miRNAs could be targeting *sparc* in ABSa15 cells, the conservation and clustering of putative miR-29 binding sites, and its relatively high expression in this cell line, indicate that miR-29 is a strong candidate to post-transcriptionally regulate seabream *sparc* in this system. Furthermore, this regulation could account for the observed phenotype upon miR-29 overexpression.

3.4.6. β-catenin protein levels are up-regulated in ABSa15 cells overexpressing miR-29

In mammals, miR-29 modulates canonical Wnt signalling through a positive feedback loop and, consequently, contributes to osteoblast differentiation (Kapinas et al., 2010). In this regulatory circuit, canonical Wnt signalling induces miR-29 transcription, which in turn potentiates this pathway by down-regulating three antagonists: Dkk1, Kemen2 and sFRP2 (Kapinas et al., 2010). Since in ABSa15 cells miR-29a was able to induce ECM mineralization and/or accelerate cell differentiation, we hypothesized that canonical Wnt signalling could also be potentiated by miR-29a thus contributing to promote ABSa15 cell differentiation towards mineralization. To test this

theory, β-catenin protein levels were assessed in the OE-miR-29a ABSa15 clone and compared to those in WT cells. Indeed, western-blot data showed that β-catenin protein levels were significantly up-regulated by approximately 40% in cells overexpressing miR-29a, demonstrating that canonical Wnt signalling is stimulated in this condition (Fig. 3.7). This result suggests that the positive feedback loop Wnt signalling/miR-29a was conserved throughout evolution. Furthermore, as in mammalian models, this mechanism is also likely to participate in osteogenic differentiation in fish.

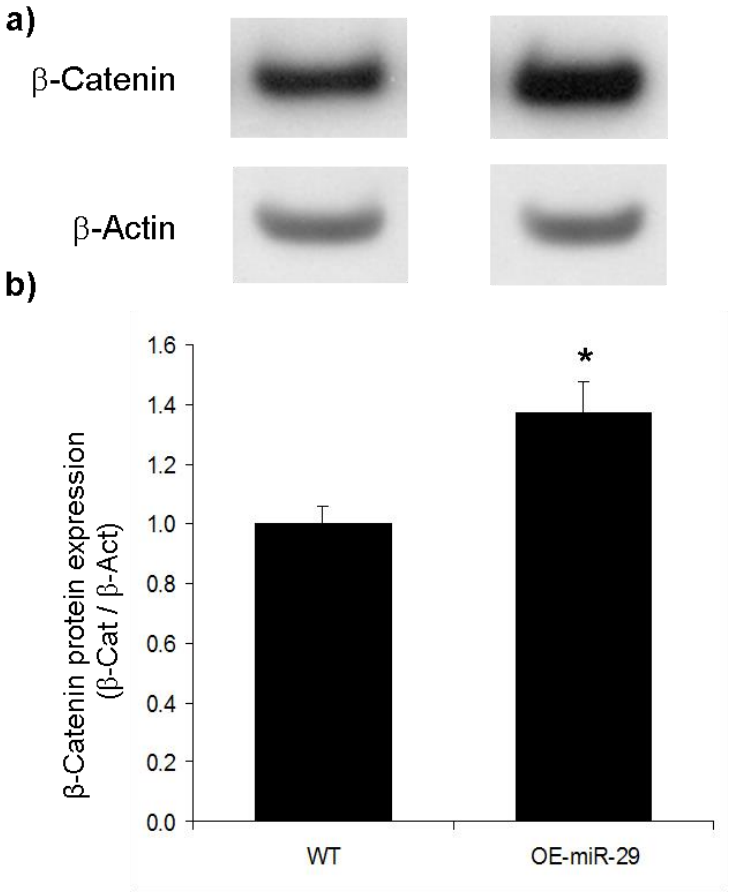

Figure 3.7. Levels of β-catenin protein production in wild type ABSa15 cells (WT) and stable clone overexpressing miR-29a (OE-miR-29). Production of β-catenin protein was determined in confluent cultures by densitometry analysis of western-blot signals (a) and normalized using β-actin signals (b). Asterisk (*) indicates value statistically different from WT. Values are the mean of at least 3 independent replicates. (Student's t-test, $p < 0.05$).

3.5. Discussion

MiRNAs have recently been implicated in skeletogenesis, through regulation of different molecules and pathways. Here, we analysed the effect of miR-29a overexpression in a skeletal derived-fish cell line and provided further insights into the conservation of miR-29 family across vertebrates, as well as in the conservation of miR-29 mechanism of action.

3.5.1. miR-29 is a generally conserved family of miRNAs

According to our analysis, sequence homology of each miR-29 family member was highly conserved among vertebrates, with higher similarity between miR-29a and miR-29c than miR-29b, in all species analysed. In fact, the 3'-end nucleotides of miR-29b, distinct from miR-29a and miR-29c, seem to be required for nuclear localization, where miR-29b was previously found to be enriched (Hwang et al., 2007; Liao et al., 2010). Conservation of miR-29b 3´-end across species suggests that this sub-cellular distribution might be evolutionary maintained. In mammals, miR-29a and miR-29c differ in one central nucleotide and, interestingly, both miR-29a and miR-29c of non-mammalian species are identical to miR-29c of mammals. This could indicate that miR-29a is specific of mammals. Strikingly, synteny analysis suggested that miR-29a of non-mammalian species is a true ortholog of mammalian miR-29a. These data raised the hypothesis that a mutation in mammalian miR-29a might have occurred, possibly after a gene duplication event, leading to the origin of a new miR-29 in mammals. Although this theory needs to be confirmed, gene duplication events succeeded by functional change/acquisition are considered a main source for emergence of novel miRNA genes (Berezikov, 2011).

Despite small differences in sequence, all members of miR-29 family shared a common seed region, suggesting a functional redundancy. Importantly, this seed region, which determines target binding and regulation, is preserved throughout evolution further suggesting a conservation of targets, and functions across species. In order to explore this putative conservation in vertebrates, the expression patterns of miR-29a were investigated in zebrafish and mouse. In both species, miR-29a levels of expression were higher in soft

tissues, but still present in calcified tissues, which corroborated previous results and showed that miRNAs belonging to the miR-29 family were expressed in several cell types and tissues, including muscle, heart, brain, lung and bone cells (Cushing et al., 2011; Kapinas et al., 2009; Ouyang et al., 2013; Rossi et al., 2013; Winbanks et al., 2011; Zhang et al., 2014).

3.5.2. miR-29a promotes ECM mineralization

In order to investigate the putative osteogenic role of miR-29 family in non-mammalian species, we have used a fish cell line (ABSa15) capable of *in vitro* mineralization. First we have analysed miR-29a expression during cell differentiation, mainly in the first 2 weeks of mineralogenic treatment, and throughout mineralization, from the second to the fourth week, as described previously (Tiago et al., 2014). MiR-29a strong up-regulation during cell differentiation and its maintenance during ECM mineralization suggested an important function in both processes. Not only this was in agreement with previous studies showing similar patterns of expression for miR-29a,c in mammalian osteoblasts (Kapinas et al., 2009, 2010; Li et al., 2009b), but also revealed a conservation of the regulatory mechanisms of this miRNA family. In order to further elucidate the role of miR-29a in ABSa15 cells, a stable clone overexpressing miR-29a was prepared. Obtaining this system, i.e. stable miR-29a overexpression, proved to be a challenge, not only in this study but also in previous reports (Chang et al., 2008; Kapinas et al., 2009). This difficulty could be associated to a probable inhibition of cell proliferation through direct repression of several cell growth regulators (Li et al., 2011b; Park et al., 2009; Zhao et al., 2010). Nevertheless, we were able to maintain this clone and treat with mineralogenic cocktail. Strikingly, after 3 weeks of treatment, we could already observe up to 4-folds induction in mineral deposition comparing with WT cells. One week later, ECM mineralization was still higher than WT cells, but this difference was attenuated, probably reflecting an end-stage of this process. These data suggested that miR-29a accelerates ABSa15 cell differentiation leading to premature mineral deposition. Although this was the first time that miR-29a was shown to directly influence ECM mineralization in a

bone-derived system, it lacked confirmation from either alternative clones or alternative methods for miRNA overexpression. Therefore, a transient overexpression of miR-29a was performed using miRIDIAN microRNA mimics, which resulted also in significant increase of mineral deposition relative to the negative control. This result confirmed that miR-29a induces ECM mineralization in ABSa15 cells, possibly by stimulating cell differentiation. Previous works in human and mouse *in vitro* models reported that transient transfections of miR-29b accelerated osteogenic differentiation (Li et al., 2009b; Trompeter et al., 2013), but data regarding overexpression of miR-29a is still missing in mammalian models. Here, not only we have show that miR-29a is also capable to induce osteogenesis, but also we show that exogenous expression of this miRNA promote differentiation as inferred by increased levels of osteogenic markers such as *bmp2*, *opn*, *tnap* and *oc2.* Elevated expression of these markers is consistent with the observed phenotype of ABSa15 cells, since they are well characterized osteogenic markers in mammalian systems (Ducy et al., 1996; Kaartinen et al., 1997; Murshed et al., 2005; Oliveira et al., 2003). Within teleosts, two different isoforms of osteocalcin have been described (Oc1 and Oc2) (Laizé et al., 2006) and, although both isoforms are able to bind calcium mineral phase of teleost bone (Cavaco et al., 2013), differences in their expression patterns suggested association of Oc1 to early mineralization events and Oc2 to mature osteoblast activity and bone formation (Bensimon-brito et al., 2012), further supporting our result on *oc2* up-regulation. Furthermore, BMP pathway is known to favour osteoblast differentiation and ECM mineralization thus partially explaining our results (Chen et al., 2012; Rafael et al., 2006; Tiago et al., 2014). Interestingly, although COL1A1 is considered to be a miR-29 target in mammalian systems (Li et al., 2009b), it was here shown to be also up-regulated in miR-29a overexpressing cells. However, a complementary sequence to miR-29 seed region was not found in the seabream *col1a1* 3´UTR and so it was not predicted here as a miR-29a target in ABSa15 cells (confirmed by searching in PITA and RNAhybrid prediction algorithms; data not shown). This information sets an important difference between fish and mammals. Another remarkable effect was the up-

regulation of *mgp* expression, which was also likely to be indirect. This protein is an inhibitor of soft-tissue calcification in mammals (Luo et al., 1997) and its expression in soft tissues from teleost fish suggested a conservation of that function (Gavaia et al., 2006; Pinto et al., 2003; Roberto et al., 2009). MGP is generally associated with the organic matrix of cartilage and bone *in vivo* in both mammals and fish (Hale et al., 1988; Price et al., 1983; Roberto et al., 2009). In ABSa15 cells, as well as in other fish bone-derived cells (Pombinho et al., 2004), MGP is also a marker for ECM mineralization (Tiago et al., 2014), and therefore its increase in cells overexpressing miR-29a is in agreement with the associated phenotype.

3.5.3. As in mammals, miR-29a is also capable to regulate SPARC and Wnt signalling in fish bone-derived cells

To further dissect the possible mechanisms of miR-29a action in ABSa15 differentiation/ECM mineralization, we briefly surveyed a possible effect on SPARC, a known target of miR-29 in mammalian osteoblast differentiation (Kapinas et al., 2009). SPARC has a key role in ECM assembly and deposition due to its binding to a number of different ECM components (Bradshaw, 2009). Controlled expression of this protein seems to be essential for normal osteoblastic differentiation and skeletogenesis in mammals (Delany and Hankenson, 2009; Delany et al., 2003; Mansergh et al., 2008) and fish (Estêvão et al., 2005, 2011; Laizé et al., 2005). In that sense, SPARC was shown to display several conserved features throughout evolution, mainly related to its protein structure, function and regulation (Laizé et al., 2005). Here, we bring data suggesting that SPARC post-transcriptional regulation should be conserved in fish. The two binding sites for miR-29 located in the proximal region of SPARC 3´UTR, previously described in mouse (Kapinas et al., 2009), were found to be strongly conserved among vertebrates, suggesting a functional regulation preserved during evolution. In seabream, luciferase functional assays further indicate that SPARC is also regulated in ABSa15 cells, most probably by miR-29a, which we show that is endogenously expressed in these cells. To support the idea that SPARC is a target of miR-29a in ABSa15

cells, we attempted to explore differences of SPARC protein levels when miR-29a was increased, and although our preliminary results point to a decrease of SPARC in OE-miR-29a cells, antibodies available at the moment were unable to provide a clear signal in fish cells (data not shown). Nevertheless, data regarding miR-29 indicates that the function of each member of this family might have some degree of redundancy and that it also regulates several different collagens and inhibitors of osteoblast differentiation (Li et al., 2009b; Liu et al., 2010; Sengupta et al., 2008), evidencing that miR-29 exerts a fine tuned regulation of this process by the control of several targets. Interestingly, miR-29 was shown to regulate and to be regulated by molecules of the most important signalling pathways in osteoblast differentiation, including bone morphogenetic proteins (BMPs) and transforming growth factor (TGF)-β (Luna et al., 2011; Qin et al., 2011), Hedgehog (Mott et al., 2010), insulin-like growth factor-1 (Smith et al., 2013) and Wnt (Kapinas et al., 2010). Particularly, Wnt/β-catenin (canonical) pathway, which is a major player in several steps of bone formation, essential for commitment of osteo-precursors, positive regulation of osteoblast proliferation, maturation and mineralization, but also important for regulation of osteoclast differentiation and activity (reviewed in Bodine, 2007; Hartmann, 2006), was shown not only to induce transcription of miR-29a, but also to be positively regulated by this miRNA. Indeed, miR-29a was proven to directly repress three antagonists of Wnt signalling, Dkk1, Kemen2 and sFRP2, in osteoblasts, thus contributing for the enhancement of this pathway and promoting osteoblastic differentiation (Kapinas et al., 2010). Here, we have shown that β-catenin, the only non-redundant and obligatory component of the canonical Wnt signalling, was significantly up-regulated in the ABSa15 cell clone overexpressing miR-29a, evidencing a stimulation of Wnt signalling. This result prompted us to hypothesize that miR-29a might be repressing Dkk1, Kemen2 and sFRP2 in ABSa15 cells, as described in mammals, thus increasing the binding of Wnt ligand to its receptors, inhibiting β-catenin phosphorilation by GSK-3β, thus increasing β-catenin levels in the cells, as we demonstrate here. This increase most likely up-regulates expression of downstream targets important for cell differentiation towards osteoblastogenesis

in ABSa15 cells ultimately contributing for an accelerated differentiation and premature/augmented mineralization (Fig. 3.8). Finally, the increased levels of β-catenin in fish cells overexpressing miR-29a, suggested that regulation of canonical Wnt signalling by miR-29a in bone is a conserved mechanism in vertebrates.

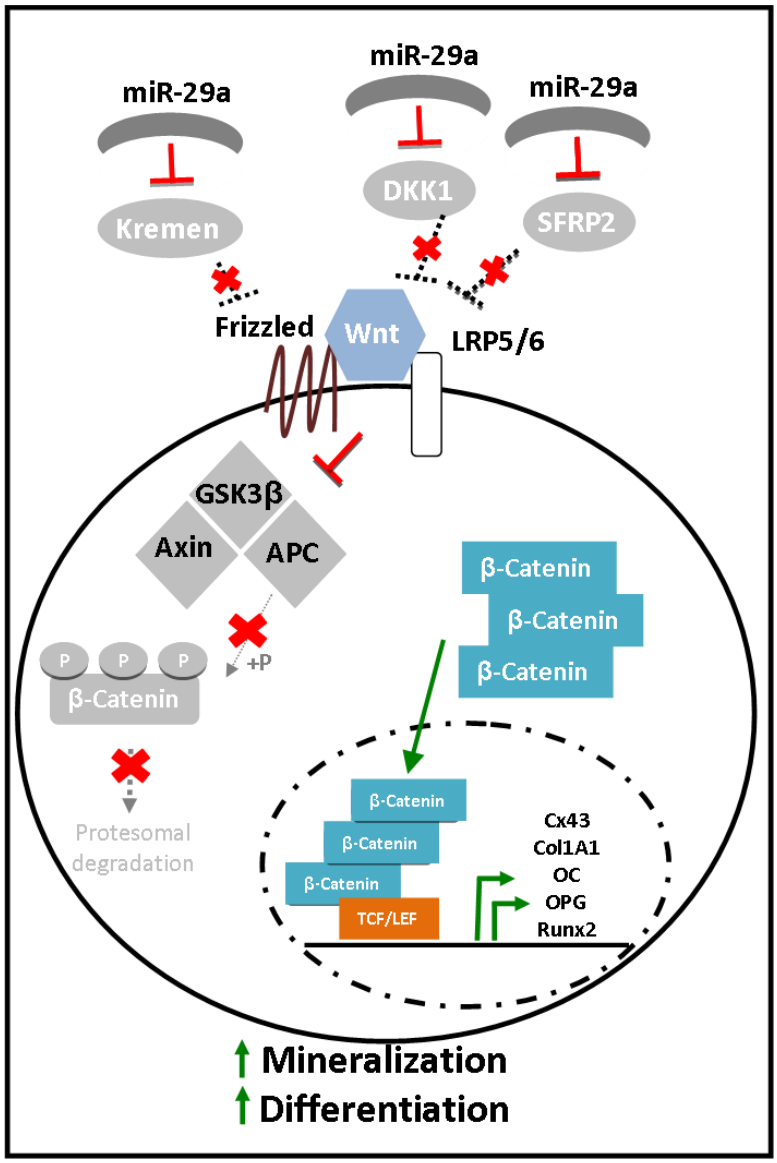

Figure 3.8. Proposed mechanism for miR-29a action on osteogenic differentiation by stimulation of canonical Wnt signalling. In the presence of miR-29a, a putative inhibition of DKK1, SFRP2 and Kremen allows the binding of Wnt ligand to its receptors (Frizzled and LRP5/6) and consequent inhibition of GSK3b/Axin/APC complex and β-catenin proteosomal degradation. β-catenin is stabilized and translocated/accumulated in the nucleus, thus activating TCF/LEF-mediated transcription of target genes such as connexin43, Col1A1, OC, OPG, Runx2, giving rise to augmented differentiation and mineralization capacity.

3.6. Conclusions

Our findings show that increased expression of miR-29a in fish bone-derived cells promotes ECM mineralization, probably by inducing cell differentiation, as reflected by stimulated canonical Wnt signalling and elevated expression of osteogenic marker genes, such as *tnap* and *oc2*. Conservation of miR-29 family sequence and organization, expression patterns and target genes further evidence a conserved function for this miRNA throughout evolution. In that sense, models other than the mammalian could help to unveil miR-29 function in bone formation as well as miR-29 function in the crosstalk between osteoblasts and osteoclasts since two recent studies demonstrated that miR-29 might also have a function in bone homeostasis (Franceschetti et al., 2013; Rossi et al., 2013). Considering these and other studies, an *in vivo* model to study miR-29 function in skeletogenesis would certainly elucidate the intricate network of genes, pathways and processes that this miRNA regulates. For this purpose, given the conservation of miR-29 regulatory action between mammals and fish, here demonstrated, and its advantages as a model organism, zebrafish seems to be a suitable model to further unveil miR-29 role in skeletogenesis.

Acknowledgements
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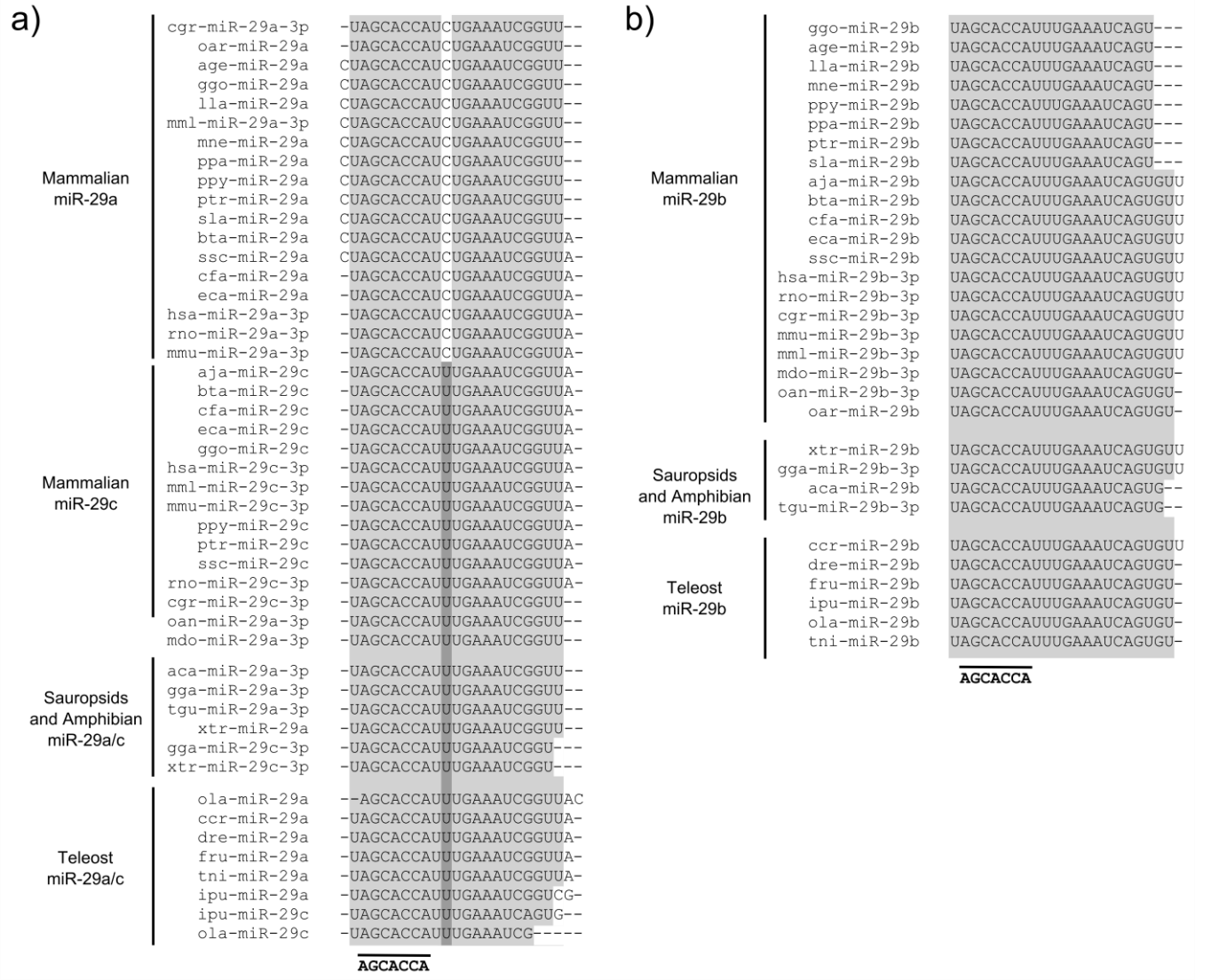
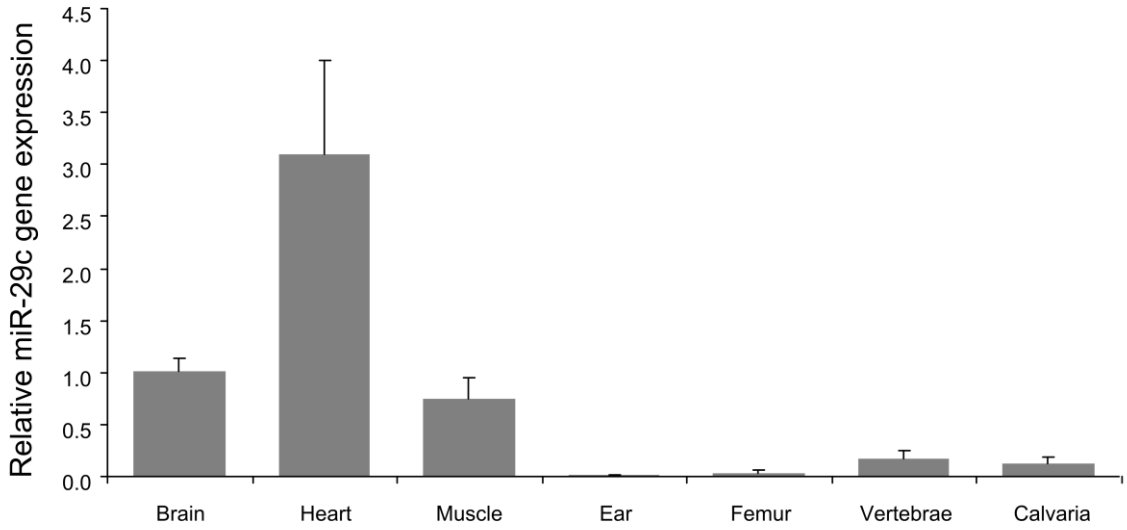
3.7. Supplementary Material

Name	Sequence
Primers used for cloning of SPARC	
Sau SPARC Fw1	5'-ATCTAAGCTCCTCAGCAGCAGCAGC-3'
Sau SPARC Rev1	5'-CCTCTGATGTAAATGTATGTTGTTGACGG-3'
qPCR primers	
Sau RPL27a Fw	5'-GGGTTACTACAAAGTTCTGGGCAAAGGC-3'
Sau RPL27a Rev	5'-TTGAAAAACACCGACAAGAGCATTATGCC-3
Sau 18S Fw	5'-CAGACAAATCGCTCCACCAACTA-3'
Sau 18S Rev	5'-CTCAACACGGGAAACCTCACC-3
Sau BMP2 Fw	5´-GCGAAGGGCATGGGCTGTCTTTGGT-3'
Sau BMP2 Ver	5'-AGCAGTACCACGAGAGAGCGGACCAC-3'
Sau β-catenin Fw	5'-TGGTTTGACACCGACCTGTAGGATTG-3'
Sau β-catenin Rev	5'-CGACTTGCTTTAACATTCCCGTGGC-3
Sau Col1a1 Fw	5´-ACGCCCTCCTGGCTTTCACCC-3'
Sau Col1a1 Rev	5´-GCCTGGTTTGGCTGGATGAAGAGGG-3'
Sau MGP Fw	5'-GTGAGGACTACTCGCCCTGCCGCTTC-3'
Sau MGP Rev	5'-CGGGAGATGCCACAGAACAACTACA-3
Sau OC1 Fw	5'-GCCTCCGCAGTGGTGAGACAGAAGAG-3'
Sau OC1 Rev	5'-TTGCTTACGTGCTTTGGGCTTGACTATAAAG-3
Sau OC2 Fw	5'-ATAATAGAATGCCGCAACAACTCCCCTGAC-3'
Sau OC2 Rev	5'-GAGATGGCGGTGATGTGGCGGAGTC-3
Sau OPN Fw	5'-AAAACCCAGGAGATAAACTCAAGACAACCCA-3'
Sau OPN Rev	5'-AGAACCGTGGCAAAGAGCAGAACGAA-3
Sau RUNX2 Fw	5'-CGGACCGACAGCCCCAACTTTCT-3'
Sau RUNX2 Rev	5'-TAGTTCTCGTCGTTGCCCGCCATA-3
Sau TNAP Fw	5'-CATCGCAACCCTTTTCACAGTCACCCG-3'
Sau TNAP Rev	5'-AACAGTGCCCAAACAGTGGTCCCATTAGC-3'
miR-29a Fw	5'-TAGCACCATTTGAAATCGGTTA-3'
Sal U6 Fw	5'-ATACAGAGAAGATTAGCATGGC-3'
Comercial primers	
oligo-d(T)-adapter primer	5'-ACGCGTCGACCTCGAGATCGATG(T)13 - 3'
universal adapter	5'-ACGCGTCGACCTCGAGATCGATG-3'
Cloning of miR-29a in pcDNA6.2-GW/EmGFP-miR	
pre-miR-29a Fw Strand	5'-TGCTGATGACTGATTTCCTTTGGTGCTTAGAGTCCCATCTGTCATCTAGCACCATTTGAAATCGGTTAT-3'
pre-miR-29a Rev Strand	5'-CCTGATAACCGATTTCAAATGGTGCTAGATGACAGATGGGACTCTAAGCACCAAAGGAAATCAGTCATC-3'

Supplementary Table 3.1. List of primers and oligoduplexes used in this study.



Supplementary Figure 3.1. Sequence alignment of mature miRNAs from miR-29 family. Sequences from mammalian, reptiles, birds, amphibian and teleost fish of (a) miR-29a/c and (b) miR-29b, were aligned using Clustal Omega. Consensus nucleotides to all species analysed are highlighted in dark grey. A distinct nucleotide in mammalian miR-29a/c is highlighted in white (C) for mammalian miR-29a and in soft grey (U) for mammalian miR-29c and miR-29a from sauropsids and teleosts. The consensus seed sequence of miR-29 family is in bold at the bottom the alignments.



Supplementary Figure 3.2. Relative miR-29c expression in mouse adult tissues. Levels of miR-29c expression were measured by miRNA specific qPCR analysis, using total RNA samples of mouse adult tissues, and normalized using U6 small RNA and brain as reference sample. Values are the mean of at least 3 independent replicates.

CHAPTER 4

miR-214: A new player in chondrogenesis?

CHAPTER 4 • miR-214: A new player in chondrogenesis?

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Submitted to Cellular and Molecular Life Sciences

4.1. Abstract

Skeletogenesis is a complex process under the control of several key transcriptional factors, hormones and signalling pathways. The regulation of this process has been greatly investigated but its post-transcriptional regulation remains generally unknown. Although microRNAs were recently shown to be important regulators of skeletogenesis, so far only few of these players have been identified and most of their targets remain unknown. Recently, miR-214 was shown to play a role in skeletal development through inhibition of osteogenesis in mammals, but data regarding other vertebrates is still scarce. A possible role in chondrogenesis remains to be demonstrated. Here, we have investigated miR-214 expression in zebrafish development and identified a possible association with skeletal formation. High expression of miR-214 in zebrafish calcified structures confirmed this hypothesis. Additionally, we showed that both human and zebrafish promoters of miR-199a-2/214 cluster are active and similarly regulated in chondrocyte cells. More importantly, overexpression of miR-214 in ATDC5 cells mitigated chondrocyte differentiation probably by targeting activating transcription factor 4 (Atf4). The key skeletal markers Matrix Gla Protein (Mgp) and osteocalcin (Oc) were simultaneously decreased upon miR-214 overexpression in ATDC5 cells, suggesting that mineralization, the late

stage of chondrocyte differentiation, might be compromised. We propose that miR-214 exerts a key role in skeletal development not only by inhibition of osteogenesis but also by affecting chondrogenesis, regulating and promoting a concerted action of important molecules for bone and cartilage formation.

4.2. Introduction

Skeletogenesis, essential in vertebrate development, is a complex process under the control of several key transcriptional factors, hormones and signalling pathways (Goldring et al., 2006; Karsenty, 2008; Véronique and Bhattaram, 2010). Although in the last two decades advancements in molecular and genetic research have uncovered diverse regulatory processes of skeleton formation, many still remain to be identified. In this context, a recently identified class of molecules belonging to the family of small non-coding RNAs, the microRNAs (miRNAs), have emerged as important regulators of various developmental, physiological and pathological processes, including the strict control of skeleton formation (Hong et al., 2012; Zhao et al., 2013). At the cellular level, miRNAs repress protein expression either by translation inhibition or by promoting messenger RNA (mRNA) degradation, both processes involving complementary binding to the 3´ untranslated region (UTR) of mRNA, mediated by the RNA-induced silencing complex (RISC). Because of this sophisticated manner of regulation, one miRNA can regulate hundreds of transcripts, whereas one mRNA can have several binding sites for distinct miRNAs. In fact, miRNAs are thought to control numerous gene regulatory networks concurrently. Moreover, as the majority of genes, miRNAs are themselves transcribed (in most cases) by RNA Polymerase II (Pol II) and therefore regulated at the transcriptional level by factors involved in multiple processes (Corcoran et al., 2009; Lee et al., 2004; Ozsolak et al., 2008). Sometimes this process involves feedback loops that further increase miRNA regulatory complexity.

Regarding skeletogenesis, miRNAs are known to participate in the major steps of bone formation either by regulating proliferation, differentiation or functional activity of cells that constitute the skeleton [9]. In this context, while

more then twenty miRNAs were identified as inhibitors or promoters of osteogenesis, only few were shown to regulate osteoclast differentiation and chondrogenesis (Lian et al., 2012). Recently, a particular miRNA previously associated to carcinogenesis, tumour progression and metastasis (Li et al., 2012; Poos et al., 2013; Yang et al., 2008), the miR-214, was shown to inhibit bone formation and it was suggested as a possible target in the treatment of osteoporosis (Wang et al., 2013a) since a negative correlation was observed between miR-214 levels and degree of bone formation in elder patients with fractures. In that study, osteoblast-specific manipulation of miR-214 levels in different mouse models revealed that miR-214 inhibitory effect was probably mediated by targeting of Atf4, a transcription factor (TF) crucial for osteoblast differentiation and function (Wang et al., 2013a). Curiously, miR-214 was for the first time implicated in skeleton formation when *Dnm3os*, the gene encoding this miRNA, was knocked-out in mice thus promoting severe skeletal defects (Watanabe et al., 2008). This transcript was then known to encode a cluster of three miRNAs, miR-214, miR-199a-2 and miR-199* (and now also including miR-214*) which are processed from two precursor stem loops, pre-miR-214 and pre-miR-199a-2 (Desvignes et al., 2014). Although all miRNAs of these cluster were then thought to contribute for the defective skeletal phenotype observed in that mouse model, only now the function of miR-214 was clearly associated to bone formation (Wang et al., 2013a). Furthermore, Lee and co-workers demonstrated that the TF Twist1 drives the expression of *Dnm3os* during mouse development (Lee and Yutzey, 2011) but, even though Twist1 plays an important role in skeleton formation of mammals and fish (Danciu et al., 2012; Reinhold et al., 2006; Yang et al., 2011b), the transcriptional regulation of *Dnm3os* has not been investigated in that process. Despite the known involvement of miR-214 in skeletal formation, data about its role in chondrogenesis is missing, although this miRNA was shown to be differentially regulated during mesenchymal stromal cells (MSCs) chondrogenic differentiation (Sorrentino et al., 2008). Therefore, understanding its involvement in chondrogenesis would contribute to further characterize the role of miR-214 in skeletogenesis. In that regard, we investigated the expression of

miR-214 in zebrafish development, from embryo to adulthood. We further explored miR-214 transcriptional regulation by studying the promoter of its primary transcript, *Dnm3os,* in human and zebrafish for the presence of conserved binding sites for TFs that are putatively involved in osteogenesis and/or chondrogenesis. More importantly, we studied the effects of miR-214 overexpression in mouse chondrocytic ATDC5 cell line unveiling, for the first time, a putative role for miR-214 in chondrocyte differentiation, *in vitro*. So, we propose that miR-214 exerts a key role in skeletogenesis not only by inhibiting osteogenesis but also by affecting chondrogenesis, regulating and promoting a concerted action of essential molecules for bone and cartilage formation.

4.3. Materials and Methods

4.3.1. Analysis of miRNAs expression

Zebrafish eggs were obtained from natural spawning of wild-type breeding fish and larvae were maintained and raised by standard methods (Westerfield, 2000). Following the Chomczynski and Sacchi method (Chomczynski and Sacchi, 1987) total RNA was extracted from a pool of up to twenty zebrafish larvae and juvenile at 1 k-cell (approximately 3 hours post fertilization, *hpf*), 18 somites (approximately 16 hpf), 24, 36 hpf, 2, 4, 6, 15, 30, 45, 60 and 81 days post fertilization (*dpf*), and from adult male and female. RNA was also isolated from different adult tissues of zebrafish specimens (muscle, branchial arches, skull and vertebra) and mice specimens (muscle, ear, femur, calvaria and vertebra). Total RNA quantity and quality were assessed by UV spectrophotometry (NanoDrop ND-1000) and agarose gel electrophoresis, respectively. For quantitative real-time PCR (qPCR) analysis of miRNAs expression, total RNA (1 µg) was polyadenylated, reverse-transcribed and amplified using miRNA-specific primers (Supplementary Table 1) using the NCode miRNA First-Strand cDNA Synthesis and NCode SYBR miRNA qPCR kits (Invitrogen), according to manufacturer's instructions. QPCR analysis was performed using the StepOnePlus system (Applied Biosystems) and relative expression of miRNAs was calculated using the ΔΔCt method (Livak and

Schmittgen, 2001) and normalized using expression levels of U6 small nuclear RNA (U6).

4.3.2. In Situ Hybridization (ISH)

Zebrafish specimens of 10, 20 and 90 dpf were euthanized with a lethal dose of MS222 (Sigma) and fixed for 24 hours (h) in 4% paraformaldehyde (PFA) at 4 ºC. Specimens were further decalcified in a 10% ethylenediaminetetraacetic acid (EDTA)/2% PFA solution for a minimum of 2 weeks and up to 2 months depending on their size. Samples were then washed in phosphate buffered saline (PBS) and maintained in 100% methanol at -20ºC until processing. For ISH, specimens were embedded in paraffin and sectioned (4-6 μm thick). Detection of dre-miR-214 was performed using an ISH protocol adapted from the method described by Kloosterman et al. (Kloosterman et al., 2006) using LNA (Locked Nucleic Acid)-modified oligonucleotide 5'-Digoxigenin (DIG) labelled probe. Briefly, sections were fixed in 4% PFA and treated with 10, 20 or 40 μg/ml of proteinase K for zebrafish specimens with 10, 20 and 90 dpf, respectively. After 2h in pre-hybridization solution (50% formamide, 5x saline sodium citrate buffer (SSC), 500 μg tRNA, 50 μg Heparin, 0.1%Tween and 9.2 mM of citric acid), sections were incubated with 40 nM of LNA ISH probe (Exiqon) specific for detection of dre-miR-214 (Supp. Table 1). For negative control, sections were hybridized with a scramble probe (Supp. Table 1). After 16 h incubation in a humidified chamber (5x SSC) at 55ºC, sections were washed with decreasing concentrations of formamide/SSC, then with PBST (PBS with 0.1% Tween-20) and incubated again for 1 h with blocking buffer (2% blocking solution from Roche diluted in Maleic Acid, 2% (v/v) sheep serum and 2% (m/v) bovine serum albumin (BSA)). Then, anti-DIG antibody conjugated with alkaline phosphatase (Roche, diluted 1:1600) was added to each section and incubated for 16h at 4ºC. Each section was washed 5 times with PBST, 3 times with AP buffer (100 mMTris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) and incubated with 75 vmg/mL NBT/50 vmg/mL BCIP in AP buffer for signal detection. Sections were air-dried, mounted with Eukitt (Sigma) and imaged by microscopy (Olympus IX-81 inverted microscope).

4.3.3. Identification and analysis of miR-214 promoter sequences

Since miR-214 is encoded in the *Dnm3os* gene, its putative promoter should correspond to the region immediately upstream the transcriptional start site (TSS) of *Dnm3os*. Although, *Dnm3os* TSS was an uncertainty for most species here analysed. Because of that, we used the known precursor sequence of miR-199a-2 (pre-miR-199a-2) as reference since it is in close proximity to the TSS of *Dnm3os*. So, pre-miR-199a-2 sequences were retrieved from miRbase database for: *Homo sapiens* (human), *Mus musculus* (mouse), *Xenopus tropicalis* (frog), *Takifugu rubripes* (fugu), *Oryzia latipes* (medaka), *Gasterosteus aculeatus* (stickleback), *Tetraodon nigroviridis* (pufferfish) and *Danio rerio* (zebrafish). Then, each pre-miR-199a-2 was blasted against the genome of each species using BLAST tool at Ensembl genome browser (www.ensembl.org) and genomic sequences with 2.5 kilobase (Kb) were collected, starting immediately upstream of pre-miR-199a-2, for further analysis. Then, a multiple sequence alignment of *Dnm3os* promoter was performed using CHAOS/DIALIGN (http://dialign.gobics.de/chaos-dialign-submission) and fed to ConTra v2 (http://www.dmbr.ugent.be/prx/bioit2-public/contrav2/) for search of putative conserved TF binding sites (TFBSs). Stringency parameters were set to: core match=0.95 and similarity matrix=0.85. Additionally, presence of conserved TFBSs in corresponding human, mouse and zebrafish sequences was also confirmed using MatInspector (Genomatix Software GmbH, Germany). The Genome Browser at UCSC (http://genome.ucsc.edu/) was used for identification of TFs previously validated through Chromatin Immunoprecipitation (ChIP assay).

4.3.4. Cloning of zebrafish miR-214 5´ end

The 5’ end of zebrafish miR-214 was achieved by rapid amplification of cDNA ends (RACE) using Advantage cDNA polymerase mix (Clontech) according to manufacturer’s conditions, and using a zebrafish Marathon cDNA library as template, previously prepared in our laboratory according to manufacture’s instructions (Clontech). Specific reverse primers (Dre Cluster

Rev1 and 2 listed in Supp. Table 1) were designed into the dre-pre-miR-199a-2 sequence available in miRbase (http://www.mirbase.org/) and combined with universal adapter primers (AP1 and AP2 universal primers; Supp. Table 1). Amplified PCR products were subsequently inserted into pCRII-TOPO (Invitrogen) and further analysed by standard DNA sequencing.

4.3.5. Plasmid Constructs

A 2.3 kb fragment and a 2.4 kb fragment of the zebrafish and the human *Dnm3os* promoter were amplified using specific primers (Dre PR Fw and Dre PR Rev, Has PR Fw and Has PR Rev, listed in Supp. Table 1) and genomic DNA as template. These PCR products were then cloned into the pCRII-TOPO vector (Invitrogen), confirmed by DNA sequencing, and further used as templates to amplify new cDNA fragments containing specific deletions of the promoter of each species. Forward and reverse primers used to amplify these fragments contained 5' ends with NheI and BglII restriction site sequences, respectively, which were used for cloning into pGL3-Basic luciferase reporter gene vector (Promega). Constructs generated in pGL3-basic were as follow: full, partial and TATA less zebrafish promoters (-1926bp/+244bp, 657bp/+244bp and -1926bp/-140bp, respectively) and full, partial and TATA less human promoters (-2299bp/+190bp, -641bp/+190bp and -2299bp/-30bp, respectively).

The pCMX-ETS1 and pCMX-TWIST1 constructs were obtained by cloning cDNA fragments of zebrafish open reading frame (ORF) of v-ets avian erythroblastosis virus E26 oncogene homolog 1 (*ets1*) (nucleotides 129-1427, GenBank accession KF774190) and *twist1a* (nucleotides 114-629, GenBank accession NM_130984.2), from 48 hpf larvae and 69 dpf juveniles respectively, into pCMX-PL2 expression vector (kindly provided by Dr. Roland Schüle, Universitats-Frauenklinik, Klinikum der Universitat Freiburg, Freiburg, Germany). BamHI and NheI restriction site sequences were incorporated into the forward and reverse primers, respectively, and the resulting PCR products were digested and cloned into the corresponding sites in pCMX-PL2 vector.

Plasmids used to express TFs in mammalian systems were kind gifts from Dr. Joseph P. Stains (Niger et al., 2011) University of Maryland, School of

Medicine, Baltimore, MD, for pcDNA3-SP1 and pcDNA3 control plasmid, from Dra. Ann Ehrlund (Pettersson et al., 2010) Karolinska Institutet, Department of Medicine, Huddinge, Stockholm for pcDNA3.1-TWIST1 and from Dr. José Bragança, CBME, University of Algarve (Bamforth et al., 2001) for pcDNA3.1-AP2α. The identity of all constructs was confirmed by DNA sequence analysis.

4.3.6. Cell culture

ABSa15 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 0.2% fungizone, and incubated at 33ºC in 10% CO_2. MC3T3-E1 and ATDC5 cells were cultured in α-MEM (supplemented with 10% FBS) and DMEM:F12 (supplemented with 5% FBS) respectively, and incubated at 37ºC in 5% CO_2. Cells were sub-cultured every 2-3 days by trypsinisation. All culture media were supplemented with 1% penicillin/streptomycin and 2 mM L-Glutamine. Cell culture media and FBS were obtained from Sigma and all other supplements and antibiotics were obtained from Gibco BRL, Gaithersburg, MD.

4.3.7. Dual-Luciferase Reporter Assays

For luciferase assays, cells were seeded in 12-well plates, further cultured for 14–16 h and transfected with X-tremeGENE HP (Roche), according to manufacturer's instructions. ABSa15 (8×10^4 cells/well) were transfected with 500 ng of luciferase construct and 300 ng of pRL-SV40 vector (Promega), ATDC5 (1×10^5 cells/well) were transfected with 500 ng of luciferase constructs and 50 ng of pRL-null vector (Promega) and MC3T3 (8×10^4 cells/well) were transfected with 500 ng of luciferase construct and 200 ng of pRL-null vector (Promega). For co-transfection experiments of TFs in ATDC5 and MC3T3, 50 ng of each construct was added to previous conditions. As control, the same amount of empty vector was added to a subsequent well. After 48 h, transfected cells were lysed and luciferase activities were measured using Dual-Luciferase Reporter Assay system (Promega) in a Synergy 4 microplate reader (Biotek). Relative luciferase activity was determined from the ratio of firefly/renilla (F-Luc/R-Luc).

4.3.8. miR-214 overexpression during ATDC5 cell differentiation

For ATDC5 transfection, cells were seeded at a density of 2.5 x 10^4 cells/ well in 24-well plates and incubated for 16 h. Subsequently, cells were transfected with miRIDIAN microRNA mimic for mmu-miR-214 (from now on designated MmiR-214) or negative control 1 (NC) (both obtained from Dharmacon) at a final concentration of 50 nM and using EzWay (Koma Biotech) transfection reagent, according to manufacturer. Then, cells were grown until confluence (T0) and differentiation was induced by supplementing medium with ITS mixture (10 μg/mL Insulin, 5.5 μg/ml transferring, 6.7 ng/ml sodium selenite, Gibco) and replaced every 2-3 days. A second transfection was performed 10 days after the first one, using the same procedure. At appropriate times, total RNA was extracted from cells using TRI Reagent (Sigma) according to manufacturer's protocol. For gene expression analysis, qPCR was performed using the StepOnePlus system (Applied Biosystems), a 1:10 dilution of MMLV-RT (Invitrogen) reverse transcribed cDNA, gene-specific primers (Supp. Table 1) and SsoFast EvaGreen supermix (Bio-Rad), according to manufacturer's instructions. miRNA qPCR analysis was performed using NCode SYBR miRNA qRT-PCR kit (Invitrogen). Relative expression was calculated using the ΔΔCt method (Livak and Schmittgen, 2002) and normalized using expression levels of hypoxanthine phosphoribosyltransferase 1 (HPRT1) for mRNA and U6 for miRNA.

4.4. Results

4.4.1. miR-214 is expressed in the skeleton of zebrafish

MiR-199a-2 and miR-214 are miRNAs that were shown to be transcribed from the opposite strand of *Dnm3,* in a common primary transcript called *Dnm3os* (Desvignes et al., 2014; Loebel et al., 2005; Watanabe et al., 2008). Conversely, this transcript was shown to be essential for normal growth and skeletal development in mice (Watanabe et al., 2008). In zebrafish, miR-214 expression was recently investigated during embryonic development (Desvignes et al., 2014; Flynt et al., 2007), but these studies failed to

demonstrate a clear association to tissue calcification. In this regard, we initiated this study by analysing miR-214 expression in several zebrafish developmental stages from blastula to adulthood, focusing in crucial stages of skeletal development. In early development, from 18 somite stage to 36 hpf, miR-214 was expressed at very low levels (Fig. 4.1). These levels dramatically increased from 2 dpf until 6 dpf (over 30-fold change comparing to 24 hpf), reaching a peak at this time period. The highest level of expression was however observed at 60 dpf (110-fold increase compared to 24 hpf). In adults, levels of miR-214 ranged from 20- to 35- fold change compared to 24 hpf and both male and female showed similar levels of expression (Fig. 4.1). Analysing miR-199a expression resulted in a similar pattern of expression (Supp. Fig. 4.1), which was consistent with the association of both miRNAs to the same transcript (cluster).

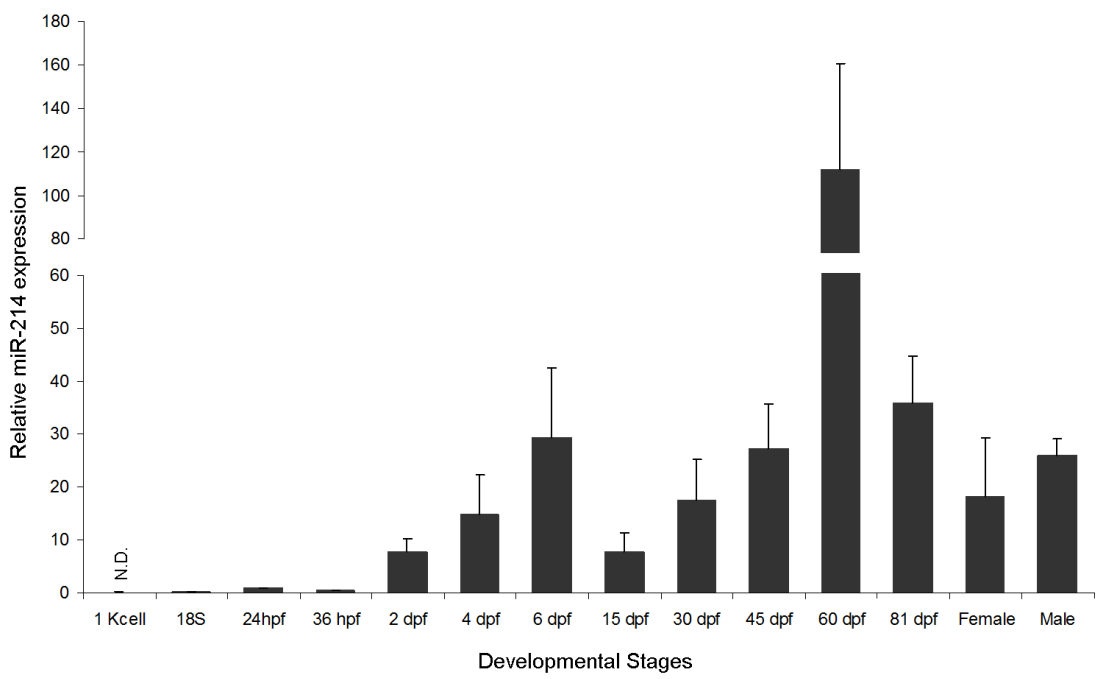

Figure 4.1. Relative expression of mature miR-214 during developmental stages of zebrafish. Levels of miR-214 expression were measured by miRNA specific qPCR analysis, using total RNA samples from different stages of zebrafish development, and normalized using zebrafish U6 small RNA and 24 hpf as reference sample. Values are the mean of at least 3 independent replicates, *hpf* hours post fertilization, *dpf* days post fertilization. *N.D.* indicates non-detected. Gap in the *y* axis separates two different scales.

In order to complement this analysis, miR-214 expression was further investigated by *in situ* hybridization in the following developmental stages: i) 10 dpf, corresponding to the onset of vertebra calcification; ii) 20 dpf, the time in which vertebra calcification is completed; and iii) 90 dpf, corresponding to young adult fish with active bone modelling. Our results show that miR-214 is widely expressed throughout the zebrafish body, being detected both in soft tissues and skeletal related tissues (Fig. 4.2). Regarding soft tissues, miR-214 was detected in several layers of the zebrafish eye, from the lens to the retina (Fig. 4.2a, b), in brain (Fig. 4.2a), in kidney (Fig. 4.2d) and in muscle (Fig. 4.2e). Regarding cartilaginous structures, miR-214 expression was evident in the chondrocranium (Fig. 4.2a, b), in the pharyngeal cartilage, in the basal region of branchial filaments (Fig. 4.2a, c), in the ceratohyal (Fig. 4.2a), and in the basis of pectoral fins (Fig. 4.2f). Furthermore, miR-214 was detected in areas of new forming bone, either in arches (Fig. 4.2h) or in vertebral bodies' growth zones (Fig. 4.2i). MiR-214 was also expressed in the notochordal sheath (Fig. 4.2g) and in the scales (Fig. 4.2e). Altogether, these results suggest, for the first time, an association of miR-214 to zebrafish skeletogenesis.

In order to get further insight into a putative association of miR-214 to calcification, miR-214 expression was investigated in several calcified tissues of zebrafish and mouse: i) branchial arches, vertebra and skull of zebrafish; and ii) ear, vertebra, calvaria and femur of mouse. Given the role of miR-214 in zebrafish myogenesis (Flynt et al., 2007), muscle was used as positive control in this analysis. Not only miR-214 was detected in the zebrafish and mouse muscle, being the tissue with the highest levels of expression, but it was also always detected in the remaining tissues of both species (Fig. 4.3). Among zebrafish calcified tissues, miR-214 levels were higher in vertebra and skull, followed by branchial arches. In mouse, all skeletal tissues analysed presented similar levels of expression except for femur, which levels were considerably lower (Fig. 4.3). These results confirm miR-214 expression in skeletal structures of zebrafish and show a conserved pattern of expression between zebrafish and mouse. This analysis suggests that, as in mouse, miR-214 might also play a role in zebrafish skeletogenesis.

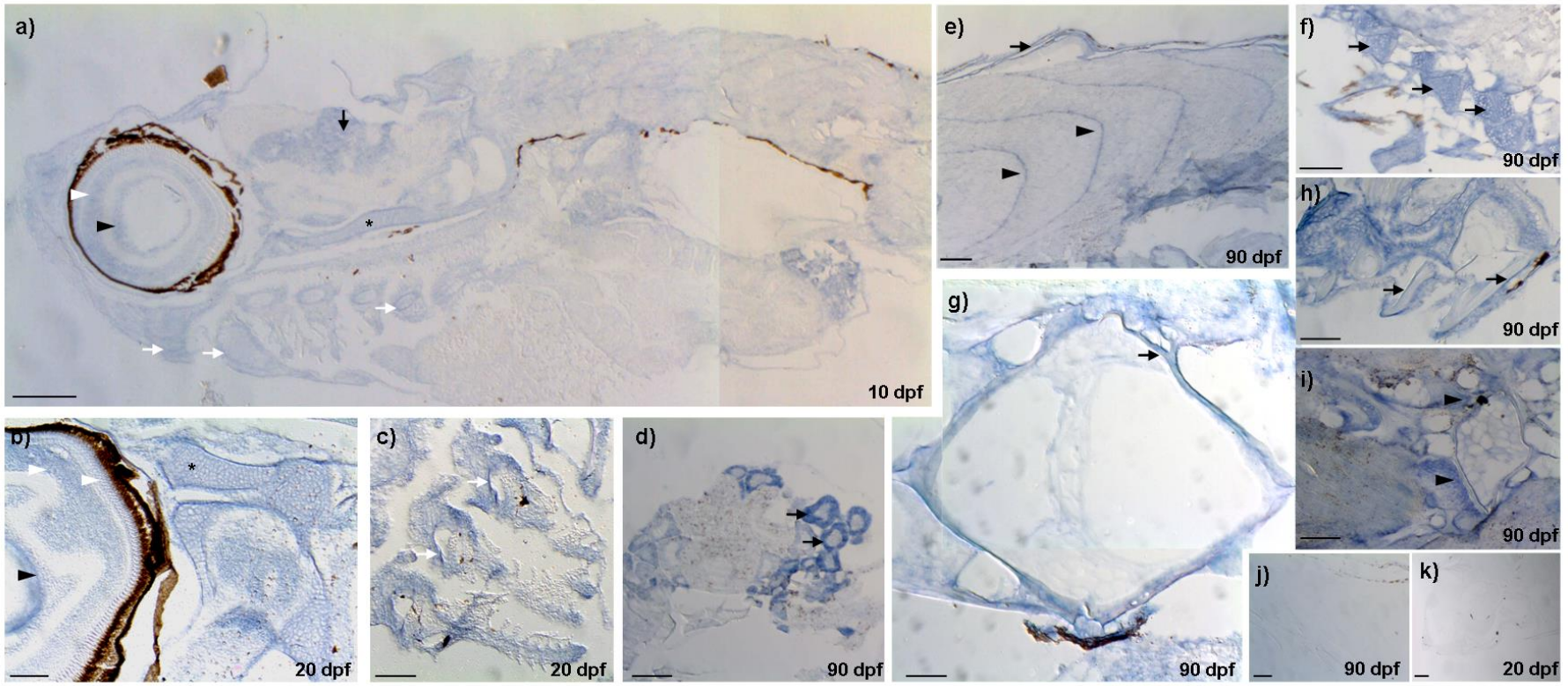

Figure 4.2. Detection of mature miR-214 in zebrafish larvae by miRNA specific *in situ* hybridization. Expression of mature miR-214 was analysed in specimens with 10 (a), 20 (b,c,d) and 90 dpf (e,f, g, h, i). From head to tail, miR-214 was detected in eye lens (arrowhead, a,b), retina (white arrowheads, a,b), brain (arrow, a), chondrocranium (asterisk, a, b), pharyngeal cartilage (white arrows, a and c), kidney (arrows, d), scales (arrow, e), muscle myotomes (arrowheads, e), cartilaginous base of pectoral fins (arrows, f), notochordal sheath (arrow, g), osteoid of haemal arches (arrows, h) and growth zones of vertebral body (arrowheads, i). Hybridization with negative control (scramble) probe did not produce detectable signal, as observed in 90 and 20 dpf specimens (j and k, respectively). Scale bars: 0.2 mm for a, e, f and k; 0.1 mm for b, c, d, h, I and j; and 0.05 mm for g.

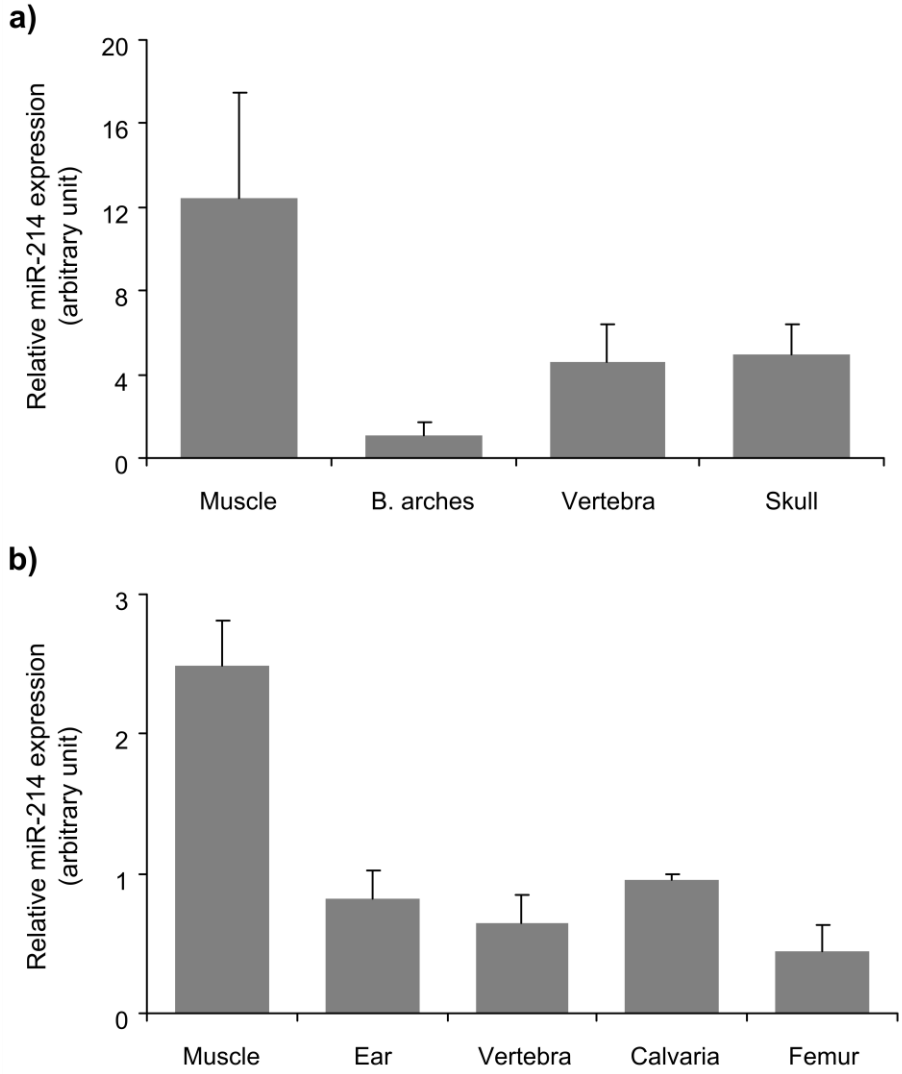

Figure 4.3. Relative expression of mature miR-214 in zebrafish (a) and mouse (b) adult tissues. Levels of miR-214 expression were measured by miRNA specific qPCR analysis, using total RNA samples of zebrafish and mouse adult tissues, and normalized using U6 small RNA. Values are the mean of at least 3 independent replicates. B. arches – Branchial arches.

4.4.2. Comparative sequence analysis of the Dnm3os putative promoter region

Although miR-199a-2/214 cluster (*Dnm3os*) is located on the opposite strand of a Dynamin 3 (*Dnm3*) intron, expression of both miRNAs and *Dnm3* gene was shown to be distinct in both zebrafish and mouse (Desvignes et al., 2014; Loebel et al., 2005). Apparently, *Dnm3os* has its own regulatory transcription unit, independent from *Dnm3*, and both in human and mouse, TWIST1 was shown to induce miR-199 and miR-214 expression through

binding to an E-box in the promoter region upstream of this cluster (Lee et al., 2009; Yin et al., 2010). So far, this regulatory mechanism was only demonstrated in mammals, and its conservation in vertebrates remains to be elucidated. Nevertheless, this cluster of miRNAs was shown to be preserved across vertebrates and to be specific of that class (Desvignes et al., 2014). In order to evaluate a possible conservation of *Dnm3os* transcriptional regulation among vertebrates, the genomic sequences upstream (~2.5 kb) pre-miR-199a of human, mouse, xenopus, medaka, stickleback, tetraodon, fugu and zebrafish were retrieved from Ensembl database and investigated. These putative promoter regions were aligned in CHAOS/DIALIGN algorithm and fed to ConTra v2 to search for conserved putative TFBS. According to this analysis, the first ~850 bp upstream of pre-miR-199a (proximal promoter) were considerably more conserved than the remaining regions, and contained most of the conserved TFBS (Fig. 4.4a). Therefore, focusing our analysis on the ~850 bp proximal promoter allowed us to identify the TWIST1 binding site previously described in human and mouse (Lee et al., 2009; Yin et al., 2010), which was found to be present/conserved in all analysed species (Fig. 4.4a, b). This indicated that the regulation by this TF might be maintained across vertebrates. The presence of TWIST1 binding site allowed us to set the parameters for subsequent analysis in ConTra v2 (core=0.95, similarity matrix=0.85), and search for conserved TFBS that could be related to chondrogenesis or osteoblastogenesis, which are functions recently associated to this cluster (Lin et al., 2009; Wang et al., 2013a; Watanabe et al., 2008), and also suggested by the profile of expression presented in the previous section (Figs. 4.1 to 4.3). From this analysis, we identified AP2alpha (transcription factor AP-2 alpha), CEBP (CCAAT/enhancer binding protein), ETS1, SP1 (Sp1 transcription factor), SRF (serum response factor) and TCF11 (also known as NFE2L1, nuclear factor, erythroid 2-like 1) as highly conserved putative binding sites in all analysed species. Moreover, a conserved non-canonical TATA box (TATAT) was identified 25 bp upstream the human TSS (GenBank accession NR_038397.2), also found to be present in seven of the eight species analysed and recognized as a putative binding site for TATA box binding protein, TBP

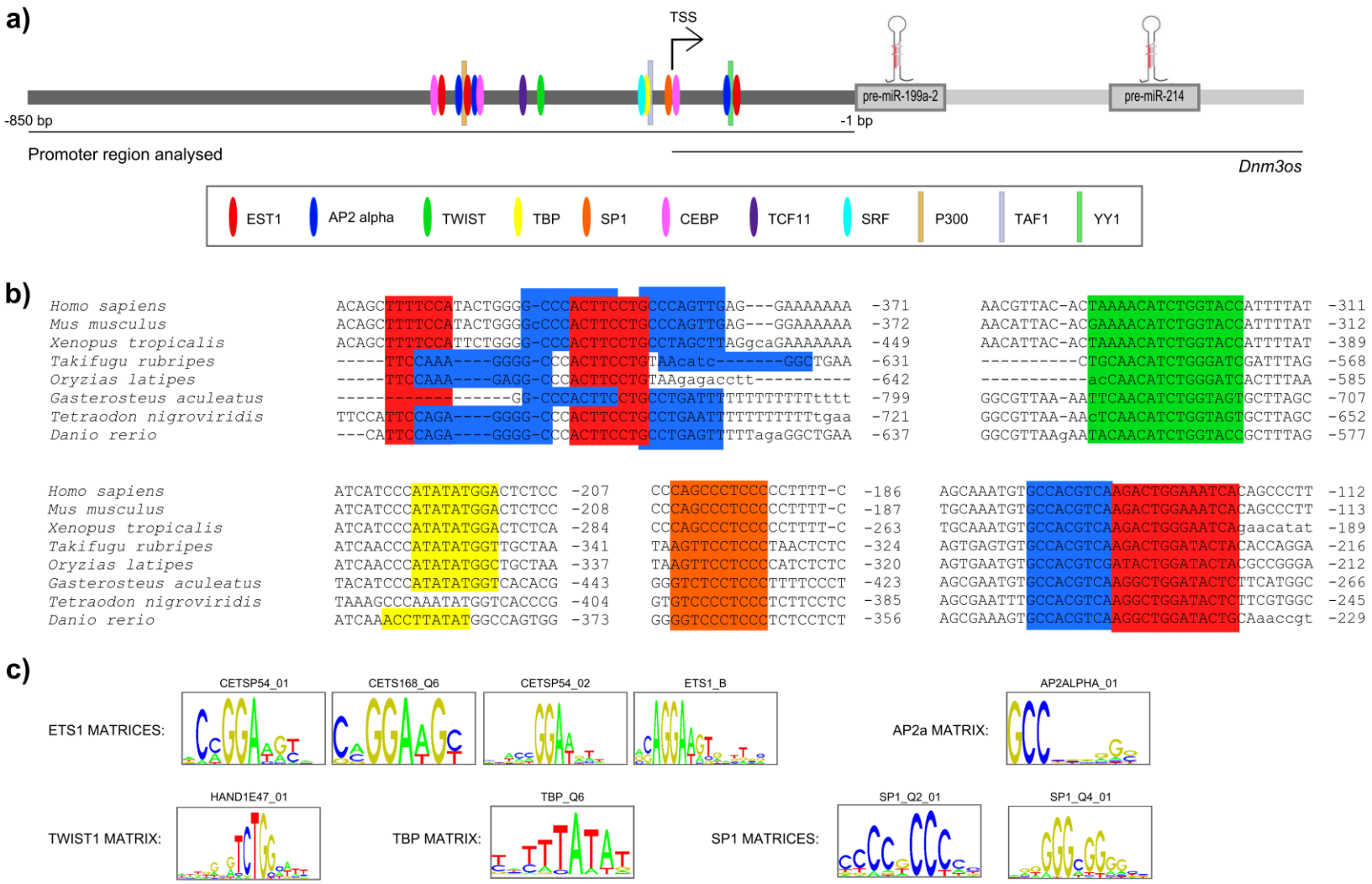
a)
TSS
pre-miR-199a-2
pre-miR-214
-850 bp
-1 bp
Promoter region analysed
Dnm3os
EST1
AP2 alpha
TWIST
TBP
SP1
CEBP
TCF11
SRF
P300
TAF1
YY1
b)
Homo sapiens
Mus musculus
Xenopus tropicalis
Takifugu rubripes
Oryzias latipes
Gasterosteus aculeatus
Tetraodon nigroviridis
Danio rerio
c)
ETS1 MATRICES:
CETSP54_01
CETS168_Q6
CETSP54_02
ETS1_B
AP2a MATRIX:
AP2ALPHA_01
TWIST1 MATRIX:
HAND1E47_01
TBP MATRIX:
TBP_Q6
SP1 MATRICES:
SP1_Q2_01
SP1_Q4_01

Figure 4.4.

Figure 4.4. Schematic representation of *Dnm3os* promoter region and identification and localization of conserved TFBS. Putative promoter sequences of *Homo sapiens*, *Mus musculus*, *Xenopus tropicalis*, *Takifugu rubripes*, *Oryzia latipes*, *Gasterosteus aculeatus*, *Tetraodon nigroviridis* and *Danio rerio* were aligned using CHAOS/DIALIGN, and then fed to ConTra v2 to search for conserved TFBS; the following parameters were used: core match=0.95, similarity matrix=0.85, TRANSFAC database. **(a)** Schematic representation of *Dnm3os* gene (not scaled) and promoter region aligned and analysed (~850 bp). Conserved TFBS predicted by ConTra v2 are indicated by colour codes. Vertical lines in colour codes indicate TFBS previously validated by ChiP assays according to UCSC Genome browser. *TSS*, Transcriptional Start Site based on human sequence. **(b)** Conserved putative binding sites for ETS1 (red), AP2alpha (blue), TWIST1 (green), TBP (yellow) and SP1 (orange) are shown. Number of the last nucleotide shown in the alignment is displayed at the right, considering that -1 nt is the first nucleotide upstream of pre-miR-199a. **(c)** Sequence logos of positional weight matrices for EST1, AP2alpha, TWIST1, TBP and SP1, as provided by ConTra v2 (http://bioit.dmbr.ugent.be/contrav2/). For TBP, parameters were: core match=0.90, similarity matrix=0.75.

(core=0.90; matrix similarity=0.75) (Fig. 4.4a, b). To further validate our comparative analysis, binding sites for three TFs (E1A binding protein p300 or P300; TAF1 RNA polymerase II TBP-associated factor or TAF1; YY1 transcription factor or YY1), previously validated by ChiP assay as regulators of human *Dnm3os*, were also found to be present in this region and shown to be conserved in the eight species analysed (Fig. 4.4a).

4.4.3. Cloning and identification of a functional Dnm3os promoter

In order to test the functionality of human and zebrafish putative *Dnm3os* promoters, the corresponding genomic regions were cloned and inserted upstream the luciferase reporter gene in pGL3-Basic vector (Promega). TSS (+1) for human was deduced based on the 5´end of the *Dnm3os* transcript variant 1 available at GenBank (NR_038397.2), whereas for zebrafish this information was not available. Therefore, we performed a 5´RACE PCR to determine the 5´end of zebrafish *Dnm3os* and obtained 4 clones of 129 bp and 2 clones of 244 bp upstream the first nucleotide of pre-miR-199a. The terminus of longest 5´end was considered as the TSS. Additionally, considering the previous *in silico* analysis showing two distinct regions in putative *Dnm3os* promoter (proximal and distal), we cloned genomic fragments corresponding to full promoters (~2.5 kb) and to partial promoters (~850 bp) in human and zebrafish. The following constructs containing putative *Dnm3os* promoters were

prepared in pGl3-Basic vector (upstream the luciferase gene): full, partial and TATA less promoters of zebrafish (-1926bp/+244bp, 657bp/+244bp and -1926bp/-140bp, respectively) and of human (-2299bp/+190bp, -641bp/+190bp and -2299bp/-30bp, respectively).

Zebrafish putative promoter constructs were transfected into a fish bone-derived cell line (ABSa15) recently developed in our lab (ECACC Ref. 13112201) (Fig. 4.5a), while human constructs were tested in MC3T3 cell line (mouse bone-derived) (Fig. 4.6a). For both species, full and partial putative promoters significantly increased luciferase expression over empty vector and, while human full and partial promoters showed similar activities (45-fold change over promoter-less vector, Fig. 4.6a), activity of zebrafish full promoter was 2 times higher then partial promoter (Fig. 4.5a). This suggests that the sequence between -1926 bp/-657 bp in the zebrafish full promoter might be regulated by activators present in ABSa15 cells. Furthermore, deletion of the putative TATA box resulted in a significant decrease of luciferase activity, similar to the levels displayed by the empty vector, in both species (Fig. 4.5a and 4.6a). This result suggested that predicted TATA box is indeed functional in both human and zebrafish. Activities of the two functional promoters, partial and full, were then assessed in ATDC5 cell line for both species. Interestingly, in this cell line, the putative full promoter increased luciferase activity to a higher extent then partial promoter for both zebrafish and human (Fig. 4.5b and 4.6b), suggesting that the distal region of these promoters, here not analysed in detail, could contain regulatory elements important for *Dnm3os* regulation in ATDC5 cell line. Also, the differences in promoter activity observed in ATDC5 and MC3T3 suggest that this cluster might be differentially regulated in distinct cell lines.

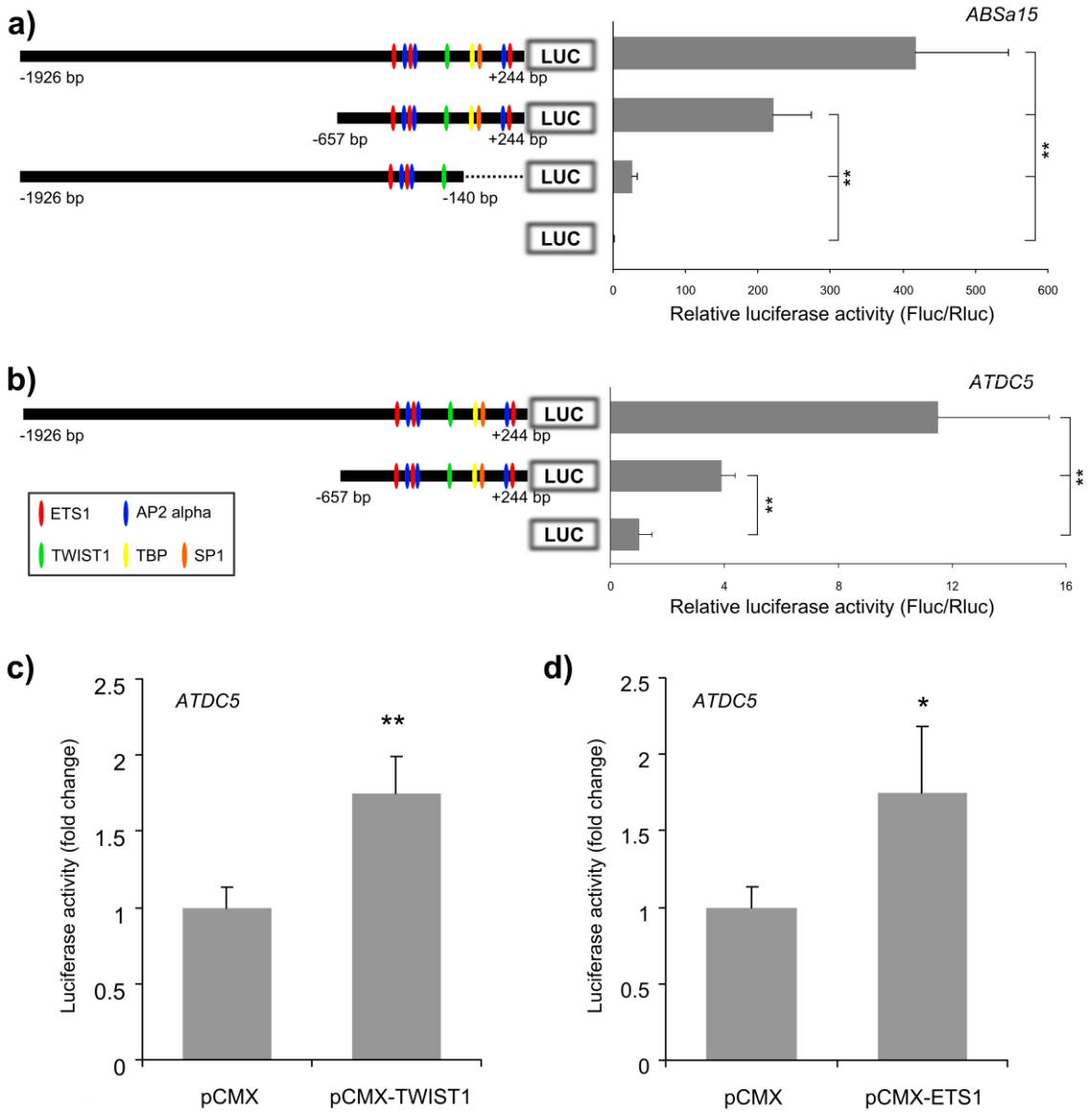

Figure 4.5. Transcriptional regulation of zebrafish *Dnm3os* putative promoter. Zebrafish full (-1926bp/+244bp) and partial (-657bp/+244bp) promoter constructs, and the promoter-less vector were transiently transfected either in ABSa15 **(a)** or ATDC5 **(b)** cells. Full promoter (-1926bp/-140bp) construct was also tested in ABSa15 cells **(a)**. A schematic representation of each construct and the respective putative TFBS (along with a legend) are indicated in the left. (Values are the mean of at least 5 independent experiments; One-way Anova, $p < 0.001$). Co-transfection of zebrafish promoter with either TWIST1 **(c)** or ETS1 **(d)** were performed in ATDC5; cells were transfected with full promoter (-1926bp/+244bp) construct (500 ng/well in 12-well plates) and either with 50 ng/well of pCMX-TWIST1, pCMX-ETS1 or empty vector together. (Values are the mean of at least 5 independent experiments; Student's t-test, * $p < 0.05$, ** $p < 0.01$). In all experiments, renilla and firefly luciferase activities were determined 48 h after transfection; in co-transfection experiments, results are indicated as fold change over the control empty vector pCMX-PL2.

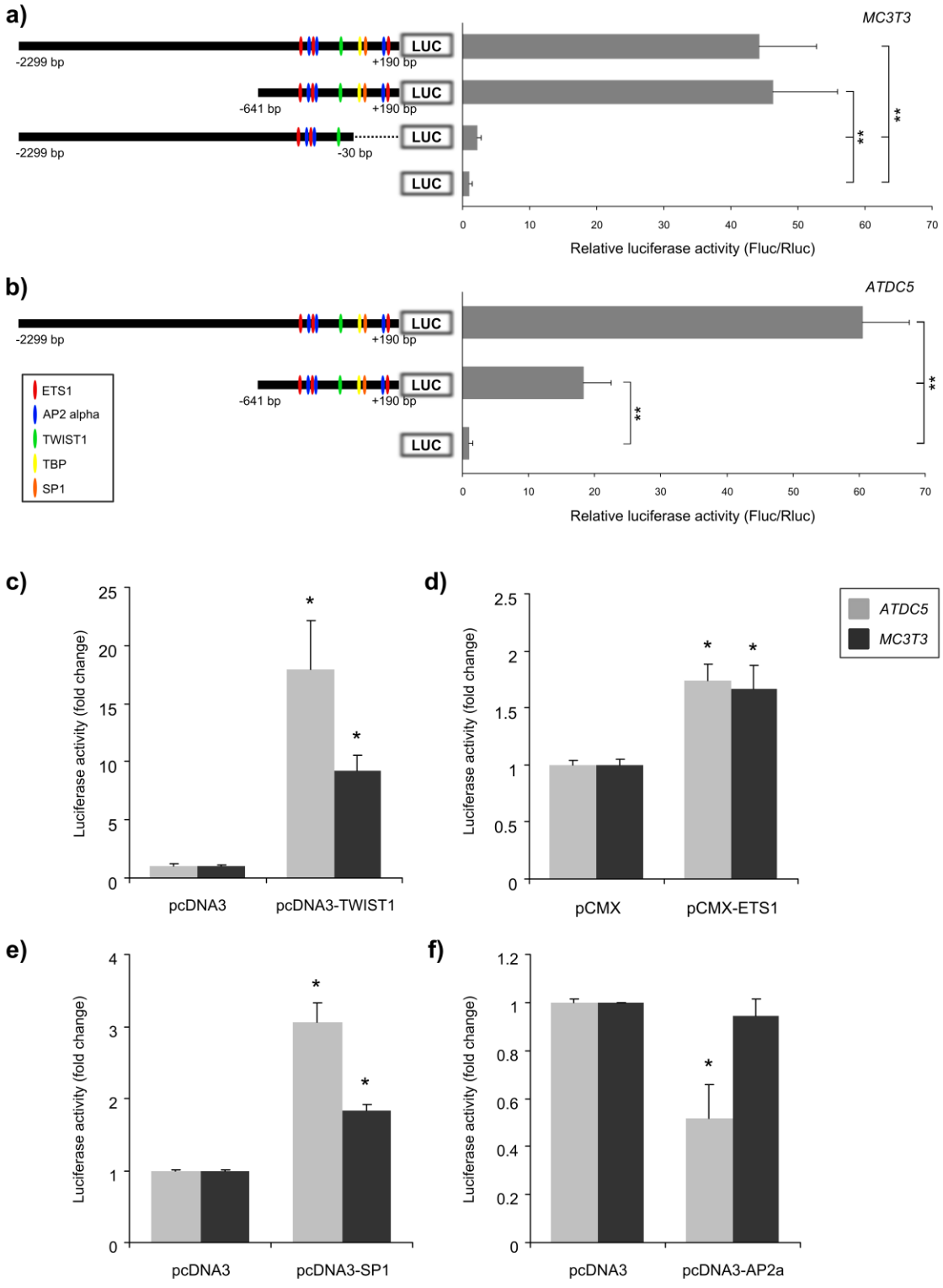

Figure 4.6. Transcriptional regulation of human *Dnm3os* putative promoter. Human full (-2299bp/+190bp) and partial (-641bp/+190bp) promoter constructs, and the promoter-less vector were transiently transfected either in MC3T3 **(a)** or ATDC5 **(b)** cells. Full promoter (-2299bp/-30bp) construct was also tested in MC3T3 cells **(a)**. A schematic representation of each construct and the respective putative TFBS (along with a legend) are indicated in the left. (Values are the mean of at least 5 independent experiments; One-way Anova, $p < 0.001$). Co-

transfection of human promoter with either TWIST1 **(c)** ETS1 **(d)** SP1 **(e)** or AP2alpha **(f)**, were performed in ATDC5 and MC3T3; cells were transfected with full promoter (-2299bp/+190bp) construct (500 ng/well in 12-well plates) and either with 50 ng/well of pCMX-TWIST1, pCMX-ETS1, pCDNA3-SP1, pCDNA3-AP2α or empty vector together. (Values are the mean of at least 3 independent experiments; Student's t-test, $p < 0.01$). In all experiments, renilla and firefly luciferase activities were determined 48 hours after transfection; in co-transfection experiments, results are indicated as fold change over the respective control empty vector pCMX-PL2 or pCDNA3. (Values are the mean of at least 3 independent experiments; Student's t-test, $p < 0.01$).

4.4.4. Transcriptional regulation of miR-214 in skeletal-related cell lines

Our *in silico* analysis retrieved several transcription factors that could regulate *Dnm3os*, and thus miR-214, in a conserved manner. TWIST1 was previously demonstrated to drive the expression of *Dnm3os*, in human and mouse (Lee et al., 2009; Yin et al., 2010) and by *in silico* analysis, we show that the previously described binding site for TWIST1 was conserved in the eight analysed species (Fig. 4.4b). In order to confirm this conservation, TWIST1 was co-transfected with human or zebrafish full promoters. These assays were performed in ATDC5 and MC3T3 cells for the human promoter (Fig. 4.6c), or just in ATDC5 cells (Fig. 4.5c) for the zebrafish promoter. We also tested zebrafish promoter in ABSa15 cells but results were inconsistent due to high variability of the data regarding renilla luciferase normalization (data not shown). For both species, TWIST1 significantly induced luciferase activity over control cells co-transfected with a vector without TF cDNA. Interestingly, co-transfection with the human promoter construct resulted in a higher increment of luciferase activity in ATDC5 (~17-fold induction) comparing to MC3T3 cells (~10-fold induction) (Fig. 4.6c). Co-transfection of TWIST1 with the zebrafish promoter resulted in a 1.7-fold induction of luciferase activity over the control (Fig. 4.5c). These results strongly suggest that *Dnm3os* transcriptional regulation by TWIST1 is preserved across species and is likely to occur in both bone and cartilage contexts.

In addition to TWIST1, several other TFs known to be involved in skeletogenesis were identified in the promoters analysed. Three of them, ETS1, SP1 and AP2alpha, were selected be also tested. Through *in silico* analysis, we identified three putative binding sites for ETS1, previously shown to be

expressed in mesenchymal precursors of newly forming bone, but never detected in the cartilage (Kola et al., 1993). While the first and third binding sites were detected by one of the eight matrices available in Contra v2 (CETSP54_02), the second binding site was detected by four matrices (Fig. 4.4b, c). Co-transfection of ETS1 with the human full promoter in MC3T3 cells resulted in a 1.7-fold induction of luciferase activity compared to the control (i.e. vector without TF cDNA) (Fig. 4.6d). Interestingly, co-transfection experiments using either the human or the zebrafish full promoters in ATDC5 cells produced similar results (Fig. 4.6d). Although these results clearly suggest a regulation of *Dnm3os* promoter region by ETS1 in both human and zebrafish, the similar effects displayed in both osteoblast and chondrocyte cells was unexpected and should be further explored in the future.

To further dissect the transcriptional regulation of *Dnm3os*, human full promoter construct was co-transfected, in ATDC5 and MC3T3 cells, with either SP1 or AP2alpha TFs, both known to play crucial roles in skeletogenesis (Ghayor et al., 2001; Niger et al., 2011; Schorle et al., 1996). While for SP1 two of the six matrices in ConTra v2 detected the same binding site, for AP2alpha we identified three conserved putative binding sites, detected by one of the four matrices at ConTra v2 (Fig. 4.4b, c). Co-transfection with SP1 significantly increased luciferase activity by 1.5-fold and 3-fold in MC3T3 and ATDC5 cells, respectively (Fig. 4.6e). Curiously, while in ATDC5 cells AP2alpha repressed *Dnm3os* promoter by 50% as determined by luciferase activity, in MC3T3 no effect was observed (Fig. 4.6f). This result suggests that *Dnm3os* transcriptional regulation might vary in different cell types, depending on the regulatory factors present. These results evidenced an involvement of different skeletal related TFs in the regulation of *Dnm3os* promoter, and indicate that this promoter contains regulatory elements that are sensitive, in a specific manner, to the factors present in different skeletal-related cell types.

4.4.5. miR-214 is down-regulated during ATDC5 chondrogenic differentiation

Recent studies demonstrated that *Dnm3os* is essential for normal skeletal development and that miR-214 inhibits osteogenesis *in vitro* and *in vivo* (Wang et al., 2013a; Watanabe et al., 2008). Although miR-214 was previously shown to be expressed in cartilage (Desvignes et al., 2014; Loebel et al., 2005; Watanabe et al., 2008), its role in chondrogenesis remains to be demonstrated. In this regard, we have used ATDC5 cells as an *in vitro* model that mimics the multistep chondrogenic differentiation occurring in endochondral bone formation (Shukunami et al., 1997; Yao and Wang, 2013), to investigate a possible role of miR-214 in chondrogenesis. Thus, we characterized miR-214 expression in critical stages of ATDC5 differentiation (Shukunami et al., 1996): i) at confluence, when cells are committed to chondrocyte lineage but are still chondroprogenitors (T0); ii) at the beginning of cartilaginous nodules formation (T9); iii) during nodule maturation, when chondrocytes are embedded in the matrix (T21); and iv) during mineralization, the later phase of differentiation (T36). According to qPCR analysis, miR-214 was highly expressed in confluent cells but strongly down-regulated (over 10-fold change) during early (T9) differentiation (Fig. 4.7).

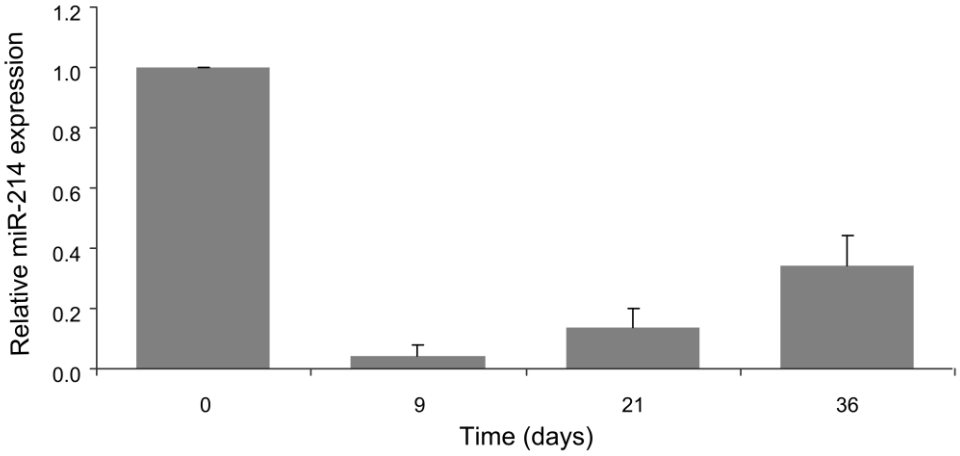

Figure 4.7. Relative expression of miR-214 during ATDC5 cell differentiation. Expression of miR-214 was determined by qPCR analysis of RNA samples collected from differentiating ATDC5 cells, and normalized using U6 small RNA expression and day 0 RNA as reference sample. Values are the mean of at least 3 independent replicates.

In subsequent stages of differentiation, miR-214 expression was somewhat increased, but its levels remained lower than T0 throughout this process: during mineralization (T36) miR-214 expression was still 6x lower than at confluent stage (Fig. 4.7). This expression pattern suggests that miR-214 levels need to be tightly controlled for chondrocyte differentiation to proceed.

4.4.6. miR-214 mitigates chondrogenic differentiation of ATDC5 cells

To investigate the functional activity of miR-214 and the relevance of its down-regulation during chondrocyte differentiation, miR-214 was transiently overexpressed in ATDC5 cells and its effects investigated through analysis of several markers of differentiation. Overexpression was achieved by transfection with miR-214 mimic (at seeding) and these experiments were simultaneously controlled by transfection with negative control mimic. When confluent, cells were cultured in the presence of ITS mixture to induce differentiation. Initially, a panel of differentiation markers was characterized in wild type (WT) cells, which where cultured and treated equally as transfected cells. Therefore, in WT cells collagen type II alpha 1 (Col2a1), collagen type X alpha 1 (Col10a1), Mgp and Oc expression levels increased during differentiation, peaking at day 28, while Sox9, alkaline phosphatase (Tnap) and Osterix (Osx or Sp7) increased until day 14 and decreased at day 28 (Fig. 4.8). These patterns of expression were in agreement with previous reports (Newman et al., 2001; Shukunami et al., 1997), which indicated that ATDC5 underwent a typical differentiation process. In addition, we have analysed the expression of Atf4, a known target of miR-214 in osteoblasts (Wang et al., 2013a), which increased during differentiation with a peak at day 28 (Fig. 4.8). Subsequently, the expression of markers of chondrogenic differentiation and miR-214 itself were assessed in ATDC5 cells overexpressing miR-214 and compared to control after 14 days of differentiation. Transfections with miR-214 mimic resulted in an average 8-fold increase over the control (Fig. 4.9a), thus confirming the delivery and presence of miR-214 mimic during the differentiation of ATDC5 cells (T14). Regarding the markers of chondrogenic differentiation, the expression levels of Col2a1, Col10a1, Tnap, Sox9 and Sp7 were not affected by miR-214 overexpression

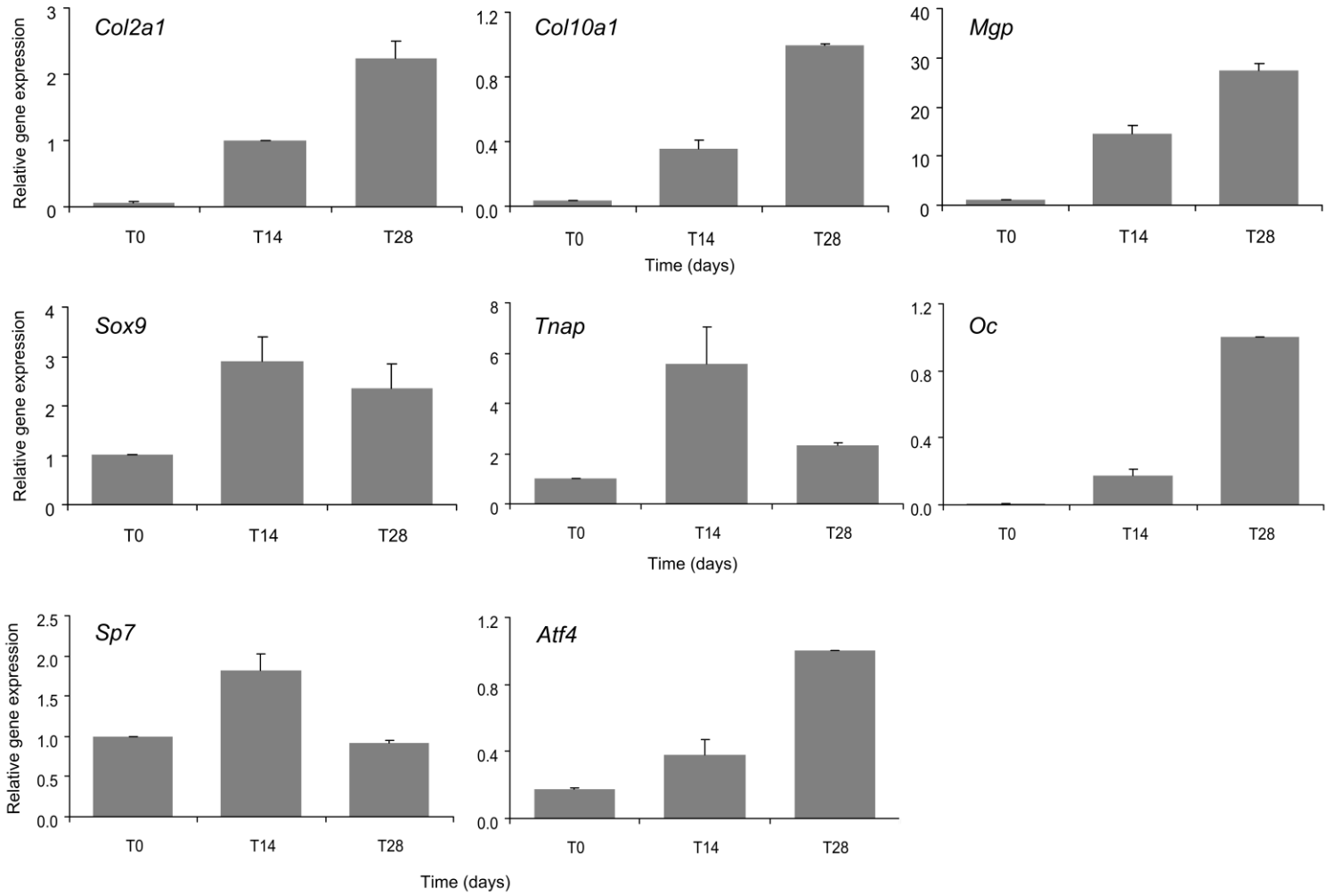

Figure 4.8. Relative gene expression of different genes associated with chondrogenic differentiation of ATDC5 cells. Expressions of type II collagen α1 (Col2a1), type X collagen α1 (Col10a1), matrix Gla protein (Mgp), sex determining region Y-box 9 (Sox9), alkaline phosphatase liver/bone/kidney (Tnap), osteocalcin (Oc), Sp7 and Atf4 were evaluated by qPCR analysis of total RNA samples collected from confluent cultures (T0) of ATDC5, and after 14 (T14) and 28 (T28) days of differentiation. Gene expression was normalized using HPRT1 housekeeping gene expression (similar expression data was collected using HPRT6 and GAPDH housekeeping genes; data not shown). Values are the mean of at least 3 independent replicates

(data not shown). On the contrary, Mgp and Oc were significantly reduced by approximately 20% and 60% respectively, in cells overexpressing miR-214 (Fig. 4.9b), suggesting that normal cell differentiation was compromised. As for miR-214 target, Atf4 expression decreased by approximately 40% after miR-214 overexpression (Fig. 4.9b). The regulation of Atf4 suggested that the miR-214 mimic introduced in ATDC5 cells was functional and probably controlling Atf4 during chondrogenesis, as it was already shown in osteogenesis (Wang et al., 2013a). According to these data, it is likely that miR-214 mitigated chondrocyte differentiation (as determined by Mgp and Oc down-regulation) by, at least in part, specifically targeting Atf4.

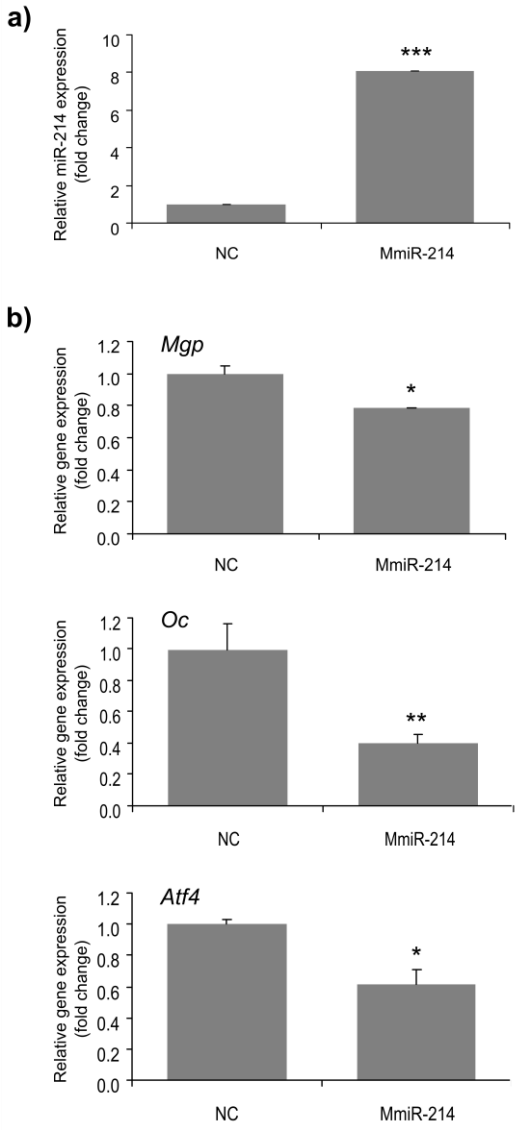

Figure 4.9. Effect of miR-214 overexpression in ATDC5 chondrocyte differentiation. Levels of miR-214 mature miRNA expression (a), and Mgp, Oc and Atf4 gene expressions (b) in ATDC5 undergoing differentiation, and transfected with mmu-miR-214 mimic (MmiR-214) or corresponding negative control (NC). MmiR-214 or NC were transfected into ATDC5 cells 16 h after seeding and differentiation was induced when cells reached confluence (T0). After 14 days of treatment, total RNA samples were collected and used to determine the expression of miR-214 by miRNA specific qPCR analysis, and normalized using U6 small RNA; alternatively, collected RNA samples were used to determine the expression of Mgp, Oc and Atf4 by common qPCR analysis and. normalized using HPRT1 housekeeping gene (similar expression data was collected using HPRT6 and GAPDH housekeeping genes; data not shown). Results are presented as fold change over NC. Asterisks indicate values statistically different from NC (Values are the mean of at least 3 independent replicates; Student's t-test, *** $p < 0.001$; ** $p < 0.01$, * $p < 0.05$).

4.5. Discussion

Skeletogenesis is still under study concerning its post-transcriptional regulators. Previous reports have shown that miR-214 is an inhibitor of mammalian bone formation (Wang et al., 2013a) and that its expression is driven by an important skeletal regulator, TWIST1 (Lee et al., 2009). Furthermore, miR-214 was shown to be vertebrate specific and evolutionarily conserved (Desvignes et al., 2014), suggesting that its function may have been maintained. In other studies, miR-214 expression was also associated to cartilaginous structures of mouse and zebrafish (Desvignes et al., 2014; Watanabe et al., 2008), although its function in chondrogenesis remains unknown. To address these issues, we studied miR-214 in zebrafish, mouse and human models, investigating its expression, its transcriptional regulation and its effect on chondrogenic differentiation.

4.5.1. miR-214 is implicated in skeleton formation of mammals and zebrafish

In zebrafish, miR-214 was initially shown to be involved in muscle cell specification through specific regulation of hedgehog signalling (Flynt et al., 2007). However, hedgehog signalling was also previously demonstrated to play key roles in both chondrogenesis and osteogenesis (Goldring et al., 2006; Mak et al., 2008), raising the hypothesis that miR-214 could also be implicated in those processes. In fact, Watanabe and co-workers showed that the primary transcript from which miR-214 is processed (along with miR-199a, miR-199* and miR-214* (Desvignes et al., 2014)), *Dnm3os*, is essential for normal skeletal development in mice (Watanabe et al., 2008). Furthermore, expression analysis in 72 hpf zebrafish embryos evidenced miR-214 expression in the mesenchyme surrounding developing skeletal elements in the craniofacial skeleton (Desvignes et al., 2014) and in somites of 1 dpf embryos (Flynt et al., 2007). In order to clarify a possible involvement of miR-214 in zebrafish skeletogenesis, its expression was here investigated throughout development. This information was tentatively correlated with particular stages of skeleton formation, and although the occurrence of other processes in parallel could not

be excluded, interesting patterns were identified. MiR-214 could not be detected at 1 K-cell stage, suggesting that it was not maternally inherited, in agreement with previous studies (Desvignes et al., 2014; Flynt et al., 2007). In fact, miR-214 expression was only detected at 18 somite stage and remained low until 2 dpf, which was comparable to data presented by Flynt and colleagues (Flynt et al., 2007). From 2 to 6 dpf miR-214 was substantially up-regulated, suggesting a particular requirement for this miRNA in these stages. In this period, zebrafish skeleton is mainly composed by cartilaginous elements and otoliths (2 dpf), whereas elements from the craniofacial skeleton start to become calcified from 3 dpf onwards (Gavaia et al., 2006). Therefore, this pattern suggests that miR-214 could be related to the beginning of skeletal calcification (mainly in the head at this stage). At 15 dpf miR-214 was strongly down-regulated and only became highly expressed at 60 dpf. At 15 dpf the majority of skeletal elements become calcified (Gavaia et al., 2006), and therefore miR-214 decrease could hypothetically be associated to a negative effect of this miRNA on mineralization, as previously shown in mammalian systems (Wang et al., 2013a). From 15 to 60 dpf, there is a progressive increase in miR-214 expression that is concomitant with bone remodelling in zebrafish (Witten et al., 2001). The recruitment and proliferation of osteogenic and chondrogenic precursors could justify this up-regulation. Interestingly, in young adults with 81 dpf, miR-214 decreased to levels similar to those detected at 6 and 45 dpf. These results suggest that miR-214 is required in higher amounts when the skeleton is being actively formed. To further understand miR-214 behaviour in zebrafish skeletogenesis, we analysed the spatial component of miR-214 by *in situ* hybridization. The three stages analysed, i.e. 10, 20 and 90 dpf, corresponds to crucial moments of skeleton formation: onset and complete vertebra calcification and active bone modelling, respectively. In these stages, miR-214 was detected in both skeletal and non-skeletal components of the zebrafish body, in agreement with previous data in mouse (Loebel et al., 2005; Watanabe et al., 2008). Regarding non-skeletal developments, we show that miR-214 is expressed in the lens of the eye and retina, consistent with data in xenopus demonstrating that miR-214 is important for controlling the

developmental timing and cell fate in retina (Decembrini et al., 2009). MiR-214 was also detected in the brain, muscle and kidney consistent with *Dnm3os* expression in mouse development (Loebel et al., 2005) and further suggesting a possible conservation of miR-214 mammalian functions in the zebrafish (Chen et al., 2010; Denby et al., 2011). Regarding skeletal elements, miR-214 was found in several cartilaginous elements, i.e. chondocranium, pharyngeal cartilage and basal region of fins, and in locations where new bone is being formed, i.e. vertebral column. *Dnm3os* was previously shown to be expressed in mouse cartilage (Watanabe et al., 2008), but to our knowledge it was never detected in zebrafish cartilage. Nevertheless, a recent study reported the presence of miR-214 in the mesenchyme surrounding craniofacial skeletal elements 72 hpf zebrafish embryos (Desvignes et al., 2014), suggesting that this miRNA could be relevant to cartilaginous structure formation. Our results are therefore the first to provide data consistent with the hypothesis that miR-214 might have a role in the formation of cartilage in larvae, juvenile and adult zebrafish. Interestingly, the detection of miR-214 in zebrafish vertebral column not only was in agreement with previous qPCR data, reinforcing the idea that miR-214 is important for onset of calcification, but it was also consistent with previous data in mammalian systems. Indeed, miR-214 was recently shown to regulate important molecules for bone formation such as Sp7 and Atf4 (Shi et al., 2013; Wang et al., 2013a). Sp7 is specifically expressed in osteoblasts and is required for bone formation (Nakashima et al., 2002). In zebrafish, its expression is associated with the formation of newly mineralized matrix (Delaurier et al., 2010), co-related with miR-214 expression here described. ATF4 plays several crucial roles in osteoblast differentiation and function of mammals (Yang et al., 2004) although, to our knowledge, its function in zebrafish remains to be established. If the regulation of these genes by miR-214, and their functions, should be maintained in zebrafish, based on miR-214 expression, this miRNA could have a major role in the maintenance of normal levels of bone formation.

Finally, we confirmed miR-214 association to bone and cartilage by quantifying miR-214 expression in several skeletal related tissues of zebrafish

and mouse origin. MiR-214 expression in calcified tissues was comparable (but still lower) to that observed in muscle, a tissue in which miR-214 was already shown to play an important role (Flynt et al., 2007). Since the patterns of expression were again similar in both organisms, it is likely that miR-214 role has been conserved throughout evolution in vertebrates.

4.5.2. Cartilage and bone related TFs seem to coordinate miR-214 transcription

Temporal and spatial expression patterns of miRNAs provide evidences to unravel their functions, but also help to clarify regulatory elements/mechanisms controlling miRNA expression. In that sense, we have analysed both miR-214 and miR-199a temporal expressions in zebrafish and concluded that they share the same pattern of expression and therefore should be subjected to similar regulatory mechanisms. These results are in agreement with previous reports in mammals and zebrafish (Desvignes et al., 2014; Lee et al., 2009; Yin et al., 2010) showing that indeed miR-214 and miR-199a have a similar expression profile. The primary transcript of these miRNAs, *Dnm3os*, is encoded in the opposite strand of an intron of *Dnm3* gene in vertebrates, although gene expression analysis indicate different sites and levels for both transcripts, characteristic of independent transcriptional regulation (Desvignes et al., 2014; Loebel et al., 2005). In our study, we identified a vertebrate conserved *Dnm3os* promoter region with approximately 2.5 kb and demonstrated that zebrafish and human promoters are active in ABSa15 and MC3T3 bone-derived cell lines, respectively. A conserved TATA box was identified through *in silico* analysis and suppression of this sequence nearly abolished promoter activity indicating that this region is crucial for transcription. Interestingly, most of the conserved regulatory sites identified *in silico* were contained within the proximal 850 bp region of these promoters. In that sense, removing the remaining distal region of the human promoter did not change the associated luciferase activity in MC3T3 cell line. However, in ABSa15 (for zebrafish) and in ATDC5 (for human and zebrafish) cell lines the largest promoter regions produced somewhat higher luciferase activities. This result

indicates that other putative TFBSs or regulatory elements upstream the 850 bp proximal region (the one studied here in detail through *in silico* analysis) might be potentiating its activity in those cell lines. In previous studies, TWIST1 was shown to drive the expression of *Dnm3os* in mammals (Lee et al., 2009; Yin et al., 2010). Here, we further show that the binding site for this TF is conserved among fish, amphibian and mammals, suggesting that TWIST1 might also regulate *Dnm3os* in other vertebrates. This was confirmed in co-transfection experiments in ATDC5 cells when Twist1 was shown to significantly increase luciferase activity associated to zebrafish *Dnm3os* promoter. As control, we also showed that TWIST1 was able to induce the human promoter activity in both ATDC5 and MC3T3 cell lines, confirming data in previous reports. However, this was the first time that TWIST1 was shown to regulate a mammalian *Dnm3os* promoter in either osteogenic or chondrogenic contexts, where TWIST1 is known to exert inhibitory functions. Indeed, Twist1 was shown to repress mammalian chondrogenesis *in vivo* (Goodnough et al., 2012) and *in vitro* (Reinhold et al., 2006) by specific regulation of either Sox9 or BMP2 pathway. Concerning osteogenesis, TWIST1 was also shown to promote repressive effects in several systems: overexpression in human osteoblast HSaOS-2 cells was shown to block osteoblast differentiation (Lee et al., 1999); Twist1 interactions with ATF4 and Runx2 specifically inhibited osteocalcin expression in osteoblast cultures (Bialek et al., 2004; Danciu et al., 2012). In a similar manner, *twist1* seemed also to control zebrafish skeletal development by specific regulation of *runx2* (Yang et al., 2011c), suggesting that TWIST1 function in skeletogenesis has been maintained in vertebrates. Since TWIST1 was shown here to positively regulate both human and zebrafish *Dnm3os* promoters in pre-osteoblast (MC3T3) and pre-chondrocyte (ATDC5) cultures, we speculate whether this regulation could in part mediate its inhibitory effects in skeleton formation. In fact, inhibition of osteogenesis by miR-214 was already demonstrated in mouse pre-osteoblasts in a process involving Atf4 repression (Bialek et al., 2004; Wang et al., 2013a). It remains to be demonstrated whether this effect could also occur in pre-chondrocytes.

In order to unravel new regulatory mechanisms of miR-214, other transcription factors identified through *in silico* analysis as being putative regulators of *Dnm3os* were tested for their effect. Concerning ETS1, we have identified three conserved binding sites that could be associated to a mild induction of luciferase activity after co-transfection in ATDC5 with both zebrafish and human *Dnm3os* promoters and in MC3T3 with only the human promoter, providing additional evidence that ETS1 may regulate the expression of this cluster in both chondrocytic and osteogenic lineages. ETS1 is a pivotal molecule in osteoblast differentiation and although expressed in all stages of differentiation, higher levels are found during proliferation stage (Raouf and Seth, 2000), suggesting a more important role in this phase. More recently, ETS1 was shown to be activated by Erk pathway during chondrogenesis, and to promote chondrogenic specification of neural crest cells (Sugiura and Ito, 2010). Combined with these studies, our results suggest that ETS1 might be important to control vertebrate skeletogenesis through a mechanism involving *Dnm3os* expression. Another TF with important regulatory functions in chondro/osteogenic differentiation is SP1. Although only one SP1 binding site was identified, our functional assays indicated a significant up-regulation of luciferase activity associated to human *Dnm3os* promoter in both ATDC5 and MC3T3 cell lines, although this induction was stronger in ATDC5. SP1, through coordination with SP3, was previously shown to selectively regulate the expression of different collagen genes in differentiating chondrocytes, potentiating Col2a1 expression and inhibiting Col10a1 (Ghayor et al., 2001; Magee et al., 2005). Furthermore, SP1 was shown to promote the expression of SOX9, a key regulator of chondrogenesis (Piera-Velazquez et al., 2007). Again, these studies combined with our results suggest that SP1 could also be implicated in the regulation of chondrogenesis (and to a lesser extent in osteogenesis according to MC3T3 data) through a mechanism likely to involve miR-214 cluster. A different regulation of *Dnm3os* promoter in osteoblast- and chondrocyte-like cells was observed in AP2alpha co-transfection experiments. Three potential AP2alpha regulatory elements, proximal to ETS1 binding sites, were found to be conserved throughout vertebrates, and co-transfection with

Dnm3os promoter resulted in an inhibitory effect on ATDC5 cell line, while no effect was observed in MC3T3. AP2 family of transcription factors have crucial roles in chondrogenesis and vertebrate skeleton development, playing a dual role either as transcriptional repressors or as activators (Wenke and Bosserhoff, 2010). AP2alpha is with no doubt linked to skeleton formation, since its knockout in mice was shown to promote defects in skeletal development (Schorle et al., 1996; Zhang et al., 1996). In particular, AP2alpha was shown to be a negative regulator of chondrocyte differentiation in mammalian systems (Davies et al., 2002; Huang et al., 2004; Wenke and Bosserhoff, 2010). In this study, the repression of *Dnm3os* promoter activity by AP2alpha in ATDC5 is somehow inconsistent, since we demonstrate that miR-214 expression levels are higher in confluent ATDC5 cells, precisely the stage where AP2alpha was shown to be higher (Huang et al., 2004). Considering this, it is likely that other TFs simultaneously present in these cells overcome this effect. In sum, although further studies are necessary to confirm and expand the regulatory network controlling *Dnm3os* (and thus miR-214) expression in osteoblasts and chondrocytes, including the analysis of additional TFs, obtained data provided novel evidences regarding the transcriptional regulation of *Dnm3os* in skeletal-related systems. These results not only demonstrate that miR-214 is important and actively regulated in pre-osteoblasts, which was already demonstrated in recent studies (Wang et al., 2013a), but also emphasize the fact that it might also be crucial in chondrogenesis. In order to test this hypothesis we have investigated miR-214 altered expression in the chondrogenic cell line ATDC5.

4.5.3. miR-214, the most recent putative regulator of chondrogenesis

MiR-214 was recently found to inhibit *in vitro* and *in vivo* bone formation, by targeting ATF4 (Wang et al., 2013a), while miR-199*, another miRNA encoded in *Dnm3os*, was identified has a negative regulator of early chondrogenic differentiation (Lin et al., 2009). Although previous studies have demonstrated the presence of miR-214 in cartilaginous associated structures of both zebrafish and mouse (Desvignes et al., 2014; Watanabe et al., 2008; Wienholds et al., 2005), its role in chondrogenesis is still not understood. In

order to investigate miR-214 function in this process we have used ATDC5, a murine chondroprogenitor cell line that mimics chondrocyte differentiation *in vitro* (Shukunami et al., 1996). Expression analysis indicated that miR-214 is differentially expressed during chondrocyte differentiation, with higher levels at the chondroprogenitor state, before differentiation, suggesting that miR-214 might be important to maintain chondrocytes in an undifferentiated condition. To further explore this possibility, we altered the expression of miR-214 in ATDC5 cells using miR-214 mimic, and evaluated its impact on the expression of selected markers of chondrogenic differentiation. Our results indicated a significant down-regulation of both Mgp and Oc, while Col2a1, Col10a1, Sox9 and Sp7 were unaffected. In differentiating ATDC5 cells (non-transfected), expression of Mgp increased at nodule formation and matrix mineralization while Oc levels were significantly increased during mineralization, which was consistent with previous reports (Idelevich et al., 2011; Newman et al., 2001). Although the role of MGP and its molecular mechanisms of action in chondrocytes are not fully understood, accumulating data indicate that this protein major function is a specific inhibition of soft-tissue calcification (Luo et al., 1997). Other putative roles are associated with regulation of proliferation and apoptosis of chondrocytes in the cartilage (Newman et al., 2001). Nevertheless, MGP is recognized as one of the main markers of chondrogenic differentiation (Luo et al., 1995) and its down-regulation in miR-214 overexpressing cells indicates that ATDC5 differentiation is compromised. Oc is another marker of differentiation but mainly synthesized by osteoblasts and associated to bone formation. However, it has been shown to be expressed also in chondrocytes and VSMC, especially when cells are undergoing mineralization (Idelevich et al., 2011). In fact, overexpression of osteocalcin in ATDC5 cells was shown to stimulate differentiation and mineralization, as well as its metabolic activity (Idelevich et al., 2011). Therefore, in our experimental conditions down-regulation of Oc (as well as Mgp decrease) upon miR-214 overexpression should represent a drawback in the differentiation process, probably with consequences at mineralization. Interestingly, precisely in the later stage of differentiation miR-214 levels was slightly increased in WT cells,

suggesting that miR-214 might have a physiological function during mineralization. In that sense, a fine control of molecules responsible for this process, such as Mgp and Oc, could contribute for a normal mineral deposition in bone or cartilage. Since bioinformatics analysis did not indicate Mgp or osteocalcin as direct targets of miR-214 (data not shown), down-regulation of these genes is probably indirect. On the contrary, the repression of Atf4 by miR-214 overexpression in ATDC5 cells is most likely direct, as it was previously shown in osteoblasts (Wang et al., 2013a). Atf4 is in fact a TF essential for the regulation of osteoblast differentiation and bone development (Yang et al., 2004) and previously shown to drive Oc, by cooperative interaction with Runx2 (Dobreva et al., 2006; Xiao et al., 2005), and Sp7, through a PTH-dependent mechanism (Yu et al., 2009). It is therefore not surprising to observe a simultaneous down-regulation of Atf4 and Oc upon miR-214 overexpression in ATDC5 cells. In fact, this suggests that the same mechanism that was observed in osteoblasts (Wang et al., 2013a) is also occurring in chondrocytes. Indeed, Atf4 was previously shown to play crucial roles not only in osteoblasts but also in chondrocytes. For example, ablation of Atf4 ($Atf4^{-/-}$) in mouse was shown to alter both proliferative and hypertrophic growth plate zones through control of Indian hedgehog (Ihh) expression (Wang et al., 2009). Regarding Mgp down-regulation in ATDC5 cells overexpressing miR-214, there are other mechanisms that could explain this effect (and also on Oc). Apparently, during skeletal development in mice, SATB2 interacts with both ATF4 and Runx2 to enhance the expression of crucial genes in skeletogenesis, including Oc (Dobreva et al., 2006). Since Runx2 was previously shown to be an important regulator of Mgp gene expression (Fazenda et al., 2010; Suttamanatwong et al., 2009), we speculate whether Atf4 repression by miR-214 could affect both Mgp and Oc simultaneously and ultimately compromise chondrogenic differentiation and mineralization (Fig. 4.10).

Finally, alternative pathways for miR-214 action in ATDC5 cells cannot be discarded. For instance, in zebrafish, miR-214 was previously shown to regulate the Hedgehog pathway through targeting of a negative regulator, Supressor of fused (Sufu), and Dispatched Homolog 2, during muscle and

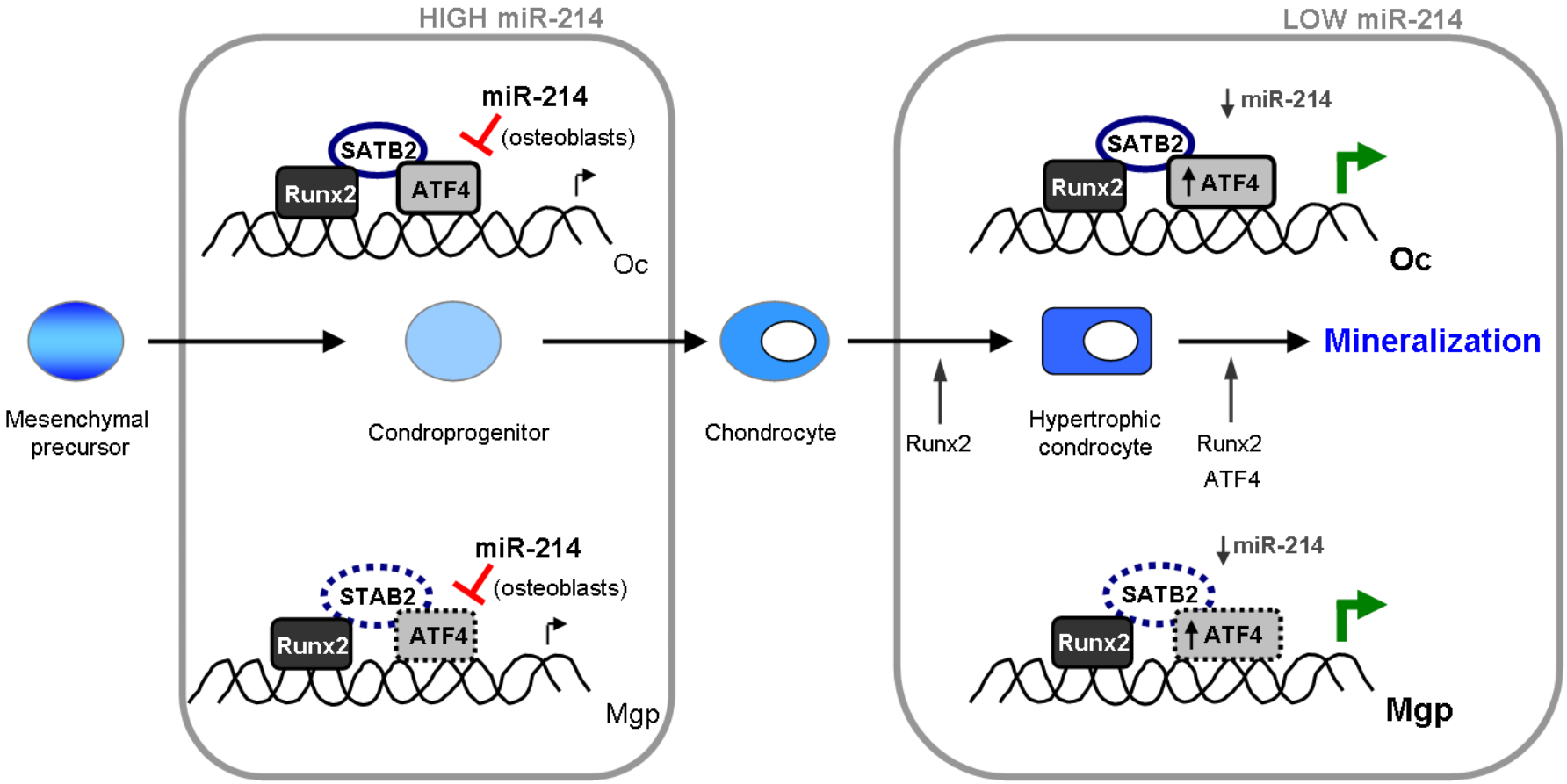

Figure 4.10. Proposed regulatory mechanism for miR-214 effect in chondrogenic differentiation. MiR-214 putatively regulates chondrogenesis by repressing ATF4, which cooperates with Runx2 and Satb2 to activate gene expression. Consequently, Oc and Mgp expression decrease, which can compromise mineralization, the latter stage of chondrogenic differentiation. Attenuated effects, in the presence of miR-214, are represented by smaller arrows. Dashed lines of SATB2 and ATF4 in Mgp promoter represent mechanisms not yet identified.

central nervous system development, respectively (Flynt et al., 2007; Li et al., 2008). Although this mechanism was not described in mammals, the regulation of this pathway is likely to occur, since putative binding sites for miR-214 are present in Sufu 3'UTR of mouse and human (data not shown). With this possibility in mind, we tested the effect of miR-214 overexpression on Hh pathway. In fact, Patched 1 levels, considered a universal marker for activation of this pathway (Murone et al., 1999), were significantly increased in ATDC5 cells overexpressing miR-214 compared to control cells (Supp. Fig. 4.2), suggesting that miR-214 could regulate this pathway also in mammals. This possibility and the regulatory mechanisms involved should be addressed in future studies.

4.6. Conclusions

In this study, we investigated several features of miR-214 concerning its involvement in skeleton formation. We showed that miR-214, a miRNA that belongs to a cluster also containing miR-199a (*Dnm3os*), has an expression pattern that is particularly associated to skeleton development and skeletal tissues in both zebrafish and mouse. Furthermore, *Dnm3os* promoter shares several conserved regulatory elements among vertebrate species, and we show that skeletal related TFs are likely to regulate this cluster in bone and cartilage *in vitro* systems. We observed that miR-214 has an interesting pattern of expression in chondrocyte ATDC5 cells, resembling that observed in osteoblast MC3T3 cells, and suggesting a similar mechanism of action in both systems. More importantly, we demonstrate that miR-214 attenuates chondrocyte differentiation of ATDC5 cell line, possibly by targeting Atf4, and ultimately decreasing the levels of both Mgp and Oc, crucial genes for normal cartilage and bone formation. These findings should be further confirmed and characterized in an *in vivo* mouse model, for instance, a specific overexpression of miR-214 in chondrocytes might answer the questions here raised.

Nevertheless, this and previous data seems to indicate that miR-214 physiological function in skeletogenesis might be to coordinate the levels of expression of key molecules involved in cell proliferation/differentiation and

bone formation, to ultimately contribute to normal skeletal development (Fig. 4.10).

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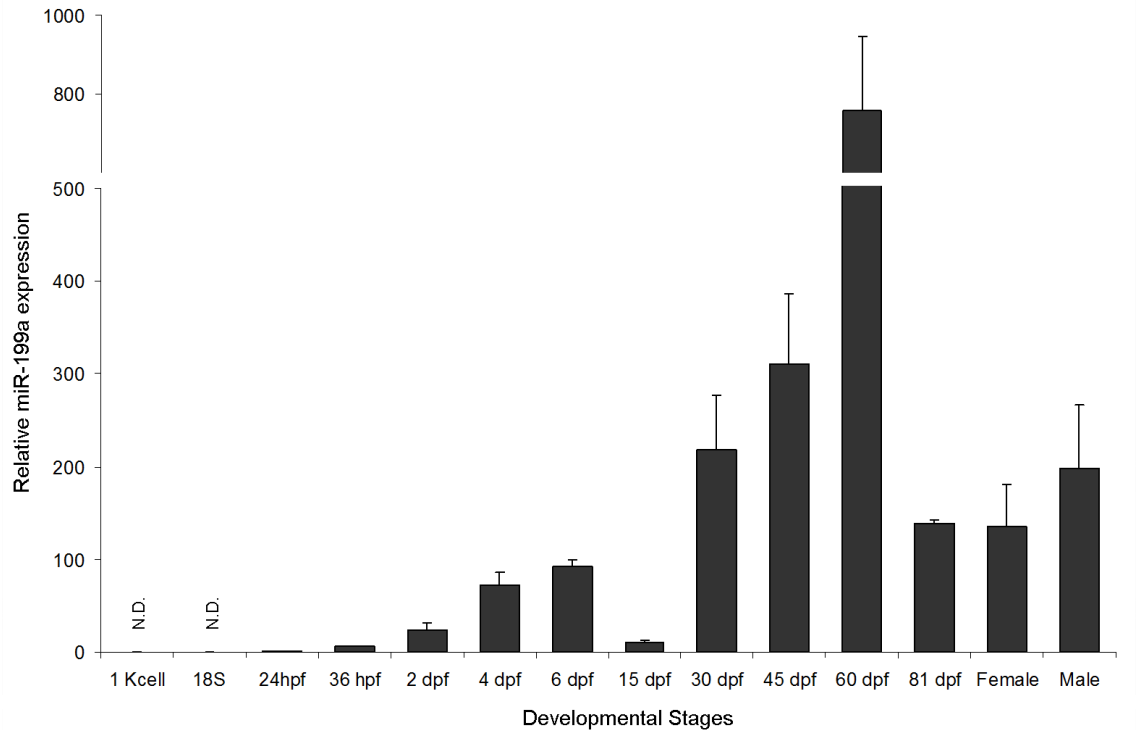
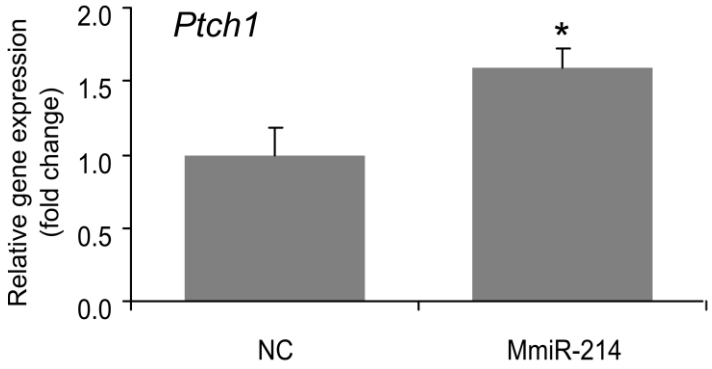
4.7. Supplementary Material

Supplementary Table 4.1. List of primers and oligoduplexes used in this study.

Name	Sequence
Primers used for cloning of zebrafish Dnm3os 5' UTR	
Dre Cluster Rev1	5'-CAGGTAGTCTGAACACTGGGATGACG-3'
Dre Cluster Rev2	5'-GATAGTTCCAGCCCTCCCTCTCTCTC-3'
Primers used for cloning of zebrafish and human promoters	
Dre PR Fw	5'-CGCATACGGTCTAGTCGCAGGAGTTC-3'
Dre PR Rev	5'-GATAGTTCCAGCCCTCCCTCTCTCTC-3'
Dre PR Fw1 NheI	5'-CGGCTAGCCGCATACGGTCTAGTCGCAG-3'
Dre PR Fw2 NheI	5'-CGGCTAGCCCTAGCGTGGACTTTACG-3'
Dre PR Rev1 BglII	5'-CGAGATCTGATAGTTCCAGCCCTCCCTC-3'
Dre PR Rev3 BglII	5'-CGAGATCTAAGGTTTGATGGGACCCTTGG-3'
Hsa PR Fw	5'-GGAGGCAAGAGCACCCACAAACACTTC-3'
Hsa PR Rev	5'-CTATGTACTTAAAATCCTCTCCCG-3'
Hsa PR Fw1 NheI	5'-CGGCTAGCCCCACAAACACTTCAGTTAAC-3'
Hsa PR Fw2 NheI	5'-CGGCTAGCGGAACTAATTACTCATGTAGA-3'
Hsa PR Rev1 BglII	5'-GAAGATCTCTATGTACTTAAAATCCTCTC-3'
Hsa PR Rev2 BglII	5'-GAAGATCTGGGATGATGTCACCCTGG-3'
Primers used for cloning of zebrafish transcription factors	
Dre Ets1a Fw	5'-GACAGCGGATCTTGTTGAGG-3'
Dre Ets1a Rev	5'-CAGTGTTGGAATGTGACTGACGC-3'
Dre Ets1a BamHI Fw	5'- CGGGATCCCACCATGACGGCAGCTGTCGATA-3'
Dre Ets1a NehI Rev	5'-CGGCTAGCTTACTCGTCCGTGTCGGG-3'
Dre Twist1a Fw	5'- GGTGATGTTTGAGGAAGAGGCGATGC-3'
Dre Twist1a Rev	5'-CCGTGCGTTAGTGAGATGTTGACATGG-3'
Dre Twist1a BamHI Fw	5'-CGGGATCCCACCATGTTTGAGGAAGAGGCGAT-3'
Dre Twist1a NehI Rev	5'-CGGCTAGCTTAGTGAGATGTTGACAT-3'
qPCR primers	
Mmu Hprt1 Fw	5´-AGCCAAATACAAAGCCTAAGATGAGCG-3'
Mmu Hprt1 Rev	5'-TCTGGGGACGCAGCAACTGACATTTC-3'
Mmu Hprt6 Fw	5'-GGTGGATATGCCCTTGACTATAATGA-3'
Mmu Hprt6 Rev	5'-CAACATCAACAGGACTCCTCCTATT-3'
Mmu Gapdh Fw	5'-CCTTCCGTGTTCCTACCCCCAATGT-3'
Mmu Gapdh Rev	5'-AGTGTAGCCCAAGATGCCCTTCAGT-3'
Mmu Tnap Fw	5'-ACAACCTGACTGACCCTTCGCTCTCCG-3'
Mmu Tnap Rev	5'-CCAGCCAAAGATGTGGAGTTGCCCCG-3'
Mmu Oc Fw	5'-AAGCAGGAGGGCAATAAGGTAGTGAACA-3'
Mmu Oc Rev	5´-GAGTTTGGCTTTAGGGCAGCACAGGTC-3'
Mmu Mgp Fw	5'-ACACAGAGGCAGACTCACAGGACACCC-3'
Mmu Mgp Rev	5'-CTGAGGGGACATAAAGGTGTTGGCAT-3'
Mmu Sox9 Fw	5'-AGGTGCTGAAGGGCTACGACTGGACG-3'
Mmu Sox9 Rev	5'-GCTGGTACTTGTAATCGGGGTGGTCTT-3'
Mmu Col2a1 Fw	5'-AAGTGGGGCAAGACCGTCATCG-3'
Mmu Col2a1 Rev	5'-AGGGGAGGACGGTTGGGTATCA-3'
Mmu Col10a1 Fw	5'-TGGGATGCCGCTTGTCAGTGCTAAC-3'
Mmu Col10a1 Rev	5'-ATCCAGGTAGCCTTTGCTGTACTCATCATA-3'
Mmu Sp7 Fw	5'-TCCTATGCTCCGACCTCCTCAACTTTT-3'
Mmu Sp7 Rev	5'-GGAAGCAGAAAGATTAGATGGCAACGAG-3'
Mmu Atf4 Fw	5'-GTGTTGGCGGGGGACTTGATGT-3'
Mmu Atf4 Rev	5'-TCTCCAACATCCAATCTGTCCCG-3'
Mmu Ptch1 Fw	5'-TGGGGGTTCTCAATGGACTGGT-3'
Mmu Ptch1 Rev	5'-CGAGTCGGAGGAATCAGACCCATT-3'
Mmu U6 Fw	5'-AGGATGACACGCAAATTCGTG-3'
Mmu miR-214 Fw	5'-ACAGCAGGCACAGACAGGCAG-3'
Mmu miR-199a Fw	5'-CCCAGTGTTCAGACTACCTGTTC-3'
Comercial primers	
oligo-d(T)-adapter primer	5'-ACGCGTCGACCTCGAGATCGATG(T)13 - 3'
universal adapter	5'-ACGCGTCGACCTCGAGATCGATG-3'
AP1 (Marathon library specic primer)	5´-CCATCCTAATACGACTCACTATAGGGC-3'
AP2 (Marathon library specic primer)	5´-ACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3'
LNA probes	
dre-miR-214 probe	DIG-5'-CTGCCTGTCTGTGCCTGCTGT-3'
Scramble probe	DIG-5'-GTGTAACACGTCTATACGCCCA-3'



Supplementary Figure 4.1. Relative expression of mature miR-199 during developmental stages of zebrafish. Levels of miR-199 expression were measured by miRNA specific qPCR analysis, using total RNA samples from different stages of zebrafish development, and normalized using zebrafish U6 small RNA and 24 hpf as reference sample. Values are the mean of at least 3 independent replicates; *hpf*, hours post fertilization, *dpf*, days post fertilization.



Supplementary Figure 4.2. Effect miR-214 overexpression on Hedgehog signalling. Patched 1 (Ptch1) expression in ATDC5 undergoing differentiation and transfected with mmu-miR-214 mimic (MmiR-214) or corresponding negative control (NC). MmiR-214 or NC were transfected into ATDC5 cells 16 hours after seeding and differentiation was induced when cells reached confluence (T0). After 14 days of treatment, collected RNA samples were used to determine the expression of Ptch1 and normalized using HPRT1 housekeeping gene (similar expression data was collected using HPRT6 and GAPDH housekeeping genes; data not shown). Results are presented as fold change over NC. Asterisk indicates value statistically different from NC (Values are the mean of at least 3 independent replicates; Student's t-test, $p < 0.01$).

CHAPTER 5

Conclusions and Perspectives

CHAPTER 5 • General Conclusions and Future Perspectives

5.1. Overview

As key regulators of gene expression, miRNAs have been implicated in a variety of physiological and pathological processes, including cell proliferation, apoptosis, differentiation, cell fate decisions, tumour progression or development (Erson and Petty, 2008; Fatica et al., 2008; Ivey and Srivastava, 2010; Wang et al., 2013b; Wienholds and Plasterk, 2005; Wu et al., 2012; Xiong et al., 2010). Emerging evidences indicate that miRNAs also play key roles in the regulation of skeletal cells differentiation, bone formation and remodelling (Kapinas and Delany, 2011; Lian et al., 2012; Zhao et al., 2013). While more then twenty miRNAs were identified as inhibitors or promoters of osteogenesis, only few were shown to regulate osteoclast differentiation and chondrogenesis (Kapinas and Delany, 2011; Lian et al., 2012; Zhao et al., 2013). In this regard, this thesis aimed at the identification and characterization of miRNAs with relevant functions in skeletogenesis. The main conclusions and possible future directions of the work are described next in separate sections, each one corresponding to a different miRNA.

5.2. miR-223 is associated to mammalian hematopoiesis and osteoclastogenesis and has conserved functions in zebrafish

In mammals, miR-223 is essential for normal myelopoiesis, promoting granulocyte, osteoclast and megakaryocyte differentiation and suppressing erythropoiesis (Haneklaus et al., 2013). However, there is a general lack of knowledge regarding miR-223 function in other vertebrates, which could help to clarify its role in other processes, such as developmental processes. At the same time, the role of miR-223 in hematopoiesis is still not fully understood, and

the identification of a research model, e.g. zebrafish, where this function has been conserved could be important for the clarification of this regulation. Therefore, our work aimed initially at analysing zebrafish as a possible and valid model to study miR-223. For that, we investigated miR-223 conservation in three different perspectives: i) from sequence to structure, ii) from gene organization to genomic context, and iii) from levels of expression to mRNA targets. Resulting data revealed that miR-223 structural and functional features have been conserved throughout evolution. Its genomic organization and gene context are maintained between human and zebrafish. In addition, we have identified 22 novel miR-223 precursor sequences and demonstrated that it contains highly conserved domains among vertebrates suggesting that processing and function of miR-223 should be also maintained in vertebrates. We also show that miR-223 patterns of expression during development are highly correlated with hematopoietic and osteoclastogenic events, providing additional evidences supporting the use of zebrafish as model to study miR-223 function. In the same manner, in adults, zebrafish miR-223 tissue distribution resembled that of mice, and it was also correlated with hematopoiesis. Finally, the miR-223 target genes that were previously associated with hematopoiesis and/or osteoclastogenesis in mammals were also predicted as putative targets in zebrafish, supporting a functional conservation of this miRNA.

In sum, our data shows that all miR-223 analysed features are generally conserved between mammals and zebrafish, indicating that the zebrafish can be an excellent model to study miR-223 role in hematopoiesis (and possibly other processes) throughout development. In future studies, miR-223 target genes involved in hematopoiesis (e.g. *lmo2*) and osteoclastogenesis (e.g. *nfia*) in zebrafish should be validated. The roles of *nfia*, *mef2c* and *igf1r* in zebrafish hematopoiesis is still not understood, and for a better understanding of this regulatory process in vertebrates, including mammals, these putative miR-223 targets should be further investigated. Interestingly, transgenic zebrafish lines, either modelling lymphoblastic leukemia (ALL) or myeloid leukemia and myeloproliferative disorder (AML/MDS) (Shen et al., 2013; Teittinen et al., 2012), are already available and could be useful tools to further explore the

regulatory mechanisms of miR-223 in leukemia. This could help to unravel the roles of *e2f1*, *stmn1*, *foxo1* and *fbxw7*, all miR-223 targets, in these pathologies.

The role of miR-223 in osteoclastogenesis is also not yet clear. Apparently, both knockdown and overexpression of miR-223 in mouse osteoclast-like cells (RAW264.7) induced osteoclastogenesis (Sugatani and Hruska, 2007, 2009), suggesting that proper levels of this miRNA are essential for this process. However, these studies did not contribute to a better understanding of miR-223 function in osteoclast differentiation and activity. In that sense, this role of miR-223 should be further addressed, probably by using *in vivo* models, such as mice transgenic models using, for instance, cathepsin k, which were proven to be useful in other studies (Mizoguchi et al., 2010; Sugatani et al., 2014). Transgenic fish in which osteoclast can be visualized in vivo (Chatani et al., 2011) could also be used as models to investigate miR-223 effect in those cells. However, osteoclastogenesis in fish has some distinct features from that of mammals, including predominantly active mononucleated vs. multinucleated cells or acellular vs. cellular bone. Therefore, interpretation and extrapolation of data between these models should be considered with extra care.

have also been used as models could also be used since *in vivo* imaging of osteoclasts in fish is possible through specific use of transgenic fish lines from medaka (but not zebrafish) in which these cells have been “labelled” with a reporter gene (Chatani et al., 2011). Although osteoclastogenesis in fish has some distinct features from the one of mammals and interpretation and extrapolation of data between models should be taken with care.

Finally, several targets of miR-223 have a function associated to angiogenesis and/or cardiovascular development in mammals and zebrafish. This prompted us to hypothesize that this miRNA could be involved in those processes Once again, zebrafish could help to clarify this possibility, since previous studies have clearly demonstrated its usefulness to investigate miRNAs involvement in vascular and heart development (Gays and Santoro, 2013).

5.3. miR-29a induces ECM mineralization in bone-derived systems through conserved mechanisms in vertebrates

The miR-29 family was previously implicated in osteoblast differentiation of mammals by targeting ECM molecules and by modulating Wnt signalling through a positive feedback loop (Kapinas et al., 2009, 2010; Li et al., 2009b). Despite all evidences concerning miR-29 effect on mammalian osteogenic differentiation, characterization of its function and regulatory mechanisms in other organisms is far from being understood, which could help to elucidate the intricate and extensive role of this miRNA family. Furthermore, the putative mineralogenic effect of miR-29 has never been demonstrated. Here, we investigated the biological effects of miR-29a overexpression in a fish bone-derived cell line, the ABSa15, capable of *in vitro* mineralization and suitable for miRNA studies (Marques et al., 2007; Tiago et al., 2014), and further explored miR-29 conservation in vertebrates.

Through this study, we provide novel insights into the biological effect of miR-29a through gain-of-function experiments in fish bone-derived cells. In ABSa15 cells, increased levels of miR-29a significantly boosted ECM mineralization, probably due to accelerated differentiation. We also demonstrated for the first time that miR-29a promotes an induction of β-catenin protein levels, implying a stimulation of canonical Wnt signalling. In addition, we show that SPARC is most likely conserved as a miR-29a target in bone-derived cells. Ultimately, miR-29a was shown to be conserved in terms of sequence homology, gene synteny and expression patterns.

In sum, miR-29a, a miRNA that was previously shown to promote osteogenic differentiation, was now demonstrated to be able to increase/accelerate mineral deposition *in vitro*, a function that seems to be conserved throughout vertebrate evolution by interaction with canonical Wnt signalling and conservation of targets. Due to this conservation, it is now clear that fish models could be useful tools to unveil miR-29 function in bone formation. For instance, miR-29 was recently suggested to participate in bone

homeostasis, but this function is not yet understood: while miR-29 was shown to promote osteoclastogenesis in primary cultures of mouse bone-marrow-derived macrophages, in RAW264.7 cell line (Franceschetti et al., 2013) it was reported to impair human osteoclast differentiation and activity (Rossi et al., 2013). It would be interesting to use fish to explore a hypothetical miRNA-mediated crosstalk between osteoblasts and osteoclasts. Considering these and other studies, an *in vivo* model to study miR-29 function in skeletogenesis would certainly elucidate the intricate network of genes, pathways and processes that this miRNA regulates.

5.4. miR-214 affects genes with crucial functions in chondrocyte differentiation and mineralization

MiR-214 was recently shown to inhibit bone formation by repressing ATF4 (Wang et al., 2013a). However, information regarding miR-214 function in other skeleton structures, including chondrogenesis, or in other vertebrates remained, until now, largely unknown. In this work, we investigated miR-214 expression, transcriptional regulation and its putative role in chondrogenesis. According to our results, miR-214 has a similar pattern of expression in zebrafish and mouse, and both are particularly associated with skeleton formation, suggesting that this miRNA role in skeletogenesis has been maintained. Regarding transcriptional regulation, the promoter of this miRNA displayed several regulatory elements that are also highly conserved among vertebrate species. Additionally, we have found that several skeletal related transcription factors are likely to regulate this miRNA in both chondrocytic and osteoblastic cells. In agreement with these results, we observed that miR-214 has a similar pattern of expression in both cell types, being highly expressed in undifferentiated cells and down-regulated during differentiation. More importantly, we demonstrated that miR-214 attenuates differentiation in chondrocytic (ATDC5) cells, probably by repression of Atf4 and also by indirectly decreasing Mgp and osteocalcin levels, both genes being crucial for normal cartilage and bone formation.

These results, combined with previous studies, indicate that miR-214 physiological function in skeletogenesis may involve the coordination of expression levels of key molecules associated with cell proliferation/differentiation and bone formation. Ultimately, this should contribute for normal bone deposition and skeletal development.

Finally, several questions that have arisen with this work should be addressed in future studies. For instance, several transcription factors binding sites have been identified in the *Dnm3os* promoter (e.g. SRF, CEBP and TCF11) and should be experimentally explored in the future. In addition, the set of transcription factors that putatively regulate the transcription of *Dnm3os* should be further validated by ChiP-assays in MC3T3 and ATDC5 cells. Also, a comprehensive characterization of miR-214 targets in ATDC5 cells should be performed in order to demonstrate the regulatory mechanisms for miR-214 effects on chondrogenesis. Although we hypothesized that cooperation between Runx2, SATB2 and ATF4 should activate the expression of both osteocalcin and Mgp in chondrogenesis (based on the mechanism already demonstrated for osteocalcin), this mechanism still has to be demonstrated. Once more, ChIP assays (possibly combined with other experiments involving specific knockdown of each transcription factor independently) could help to unveil this mystery. This would definitely increase the present knowledge concerning Mgp transcriptional regulation in vertebrates.

5.5. Concluding Remarks

MiRNAs are thought to have emerged very early in evolution. Preservation of their functions might be one of the most powerful indications for their relevance in cell function and tissue formation. Furthermore, they represent an asset for future usage as possible therapeutic resources in disease. Importantly, this study evidenced that the three miRNAs here studied in detail have conserved functions across vertebrates and, more striking, conserved mechanism of action in achieving those functions. This becomes extremely important for disease treatment research, since screening of

therapeutic drugs targeting conserved pathways can take advantage of zebrafish, which is an excellent model for drug screening.

CHAPTER 6

References

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ANNEX I

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ANNEX I • Mir-20a regulates in vitro mineralization and BMP signaling pathway by targeting BMP-2 transcript in fish

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Abstract

MicroRNAs (miRNAs) are important regulators of vertebrate development but their role during skeletogenesis remains unknown. In this regard, we investigated the mineralogenic activity of miR-20a, a miRNA associated with osteogenesis, in fish bone-derived cells. Expression of miR-20a was up-regulated during differentiation and its overexpression inhibited mineralization, suggesting a role in fish tissue calcification. In this regard, a conserved miR-20a binding site was identified in bone morphogenetic protein 2 (BMP-2) 3'UTR and its functionality was evidenced through luciferase assays, and further confirmed by western-blot and qPCR. Type II BMP receptor (BMPR2) is also targeted by miR-20a in mammalian systems and evidence was collected for the presence of a binding site in fish sequences. We propose that miR-20a is a regulator of BMP pathway through specific action on BMP-2 and possibly BMPR2. Overexpression of miR-20a was also shown to up-regulate matrix Gla protein (MGP) transcript, a physiological inhibitor of calcification previously found to form a complex with BMP-2. We propose that MGP may play a role in the anti-mineralogenic effect promoted by miR-20a by decreasing availability of BMP-2.

This study gives new insights into miRNA-mediated regulation of BMP-2, and sheds light into the potential role of miR- 20a as a regulator of skeletogenesis.

Introduction

Skeletogenesis is a complex process [1,2] and many key players and cellular mechanisms still remain to be identified. In this regard, the post-transcriptional regulation of skeletal genes has been largely under-studied and data on the skeletogenic and osteogenic role of microRNAs (miRNAs) are scarce. MiRNAs are small non-coding RNAs of ~22 nucleotides that bind to target mRNAs preventing their translation or promoting their degradation [3]. Through their post-transcriptional activity, miRNAs have been shown to regulate a broad range of biological processes [4], including skeletogenesis as evidenced by defective bone and cartilage formation resulting from conditional inactivation of DICER (the enzyme processing pre-miRNA into mature miRNA) in mouse osteoprogenitor cells [5] and in chondrocytes [6]. MiRNAs were also shown to specifically affect in vitro differentiation of chondrocytes [7], osteoblasts [5,8–10] and osteoclasts [11]. Among those miRNAs, miR-20a was recently identified as capable of promoting bone cell differentiation by targeting antagonists of the bone morphogenetic protein (BMP) 1 signaling pathway in human mesenchymal stem cells [12]. BMP pathway participates in osteoblast differentiation and plays a major role in the development of skeletal tissues (a mechanism that was shown to be conserved from fish to mammals [13,14]). Interestingly, a recent study reported the targeting of intermediates of the BMP signaling by miR-20a in biological systems not related to bone or cartilage [15,16]. Available results indicate that miR-20a may regulate BMP signaling pathway through direct and indirect mechanisms and indicates that mechanisms for miR-20a action on bone formation are far from being understood. Because they share significant similarities with mammals in organ/tissue development, bony fish represent a suitable alternative to mammals to investigate mechanisms associated with vertebrate development [17], in particular skeletogenesis [18]. The conservation of miRNA-related mechanisms throughout vertebrate evolution [19–21] also indicates the

suitability of bony fish in vivo and in vitro models to investigate the role of miRNA during skeletogenesis/osteogenesis. In this work, the ABSa15 cell line – developed from calcified branchial arches of the marine teleost gilthead seabream (*Sparus aurata*, Linnaeus, 1758) and capable of in vitro mineralization [22] – was used to investigate the post-transcriptional regulation of two key player of BMP signaling pathway by miR-20a. Data collected provided evidence for the role of miR-20a in the regulation of skeleton development, thus demonstrating the suitability of fish systems to study mechanisms of post-transcription.

Materials and methods

Cell culture and extracellular matrix mineralization

ABSa15 is a cell line previously developed from calcified branchial arches of the marine teleost gilthead seabream (*Sparus aurata*, Linnaeus, 1758) that is capable of *in vitro* mineralization [22], and was recently deposited in the European Collection of Cell Cultures (Ref. 13112201; see also Supplementary Fig. S1). ABSa15 were cultured at 33 ºC in a humidified 10% CO2 atmosphere in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), as described previously for VSa13 and VSa16 cells [23]. Human Embryonic Kidney 293 (HEK 293) cells were cultured at 37 ºC in a humidified 5% CO2 atmosphere in DMEM supplemented with 10% FBS. For mineralization experiments, ABSa15 cells were seeded in 24-well plates at 2 x 10^4 cells/well and allowed to proliferate for 1 week. Then, extracellular matrix (ECM) mineralization was induced in confluent cultures by supplementing medium with 50 lg/ml of L-ascorbic acid, 10mM b-glycerophosphate and 4mM $CaCl_2$. At appropriate times, mineral deposition was revealed through von Kossa staining and quantified by densitometry analysis [23]. Culture medium was renewed twice a week.

RNA extraction and gene expression analysis

Total RNA was extracted from cell cultures as described by Chomczynski and Sacchi [24] and quantified by UV spectrophotometry (NanoDrop ND-1000, Thermo Scientific, Madison, WI, USA). Quantitative real-time PCR (qPCR) analysis of miRNAs and mRNAs was performed using the StepOnePlus system (Applied Biosystems, Invitrogen, Grand Island, NY, USA). For qPCR analysis of mRNA expression, total RNA (1 lg) was treated with RQ1 RNase-free DNase (Promega), then reverse-transcribed using MMLV-RT (Invitrogen) and oligo-d(T)-adapter primer (Supplementary Table S1). PCR amplifications were performed using 10 ng of cDNA, gene-specific primers (Supplementary Table S1) and SsoFast EvaGreen Supermix (Bio-Rad) according to manufacturer instructions. For qPCR analysis of miRNA expression, total RNA (1 lg) was polyadenylated and reverse-transcribed using NCode miRNA First-Strand cDNA Synthesis kit (Invitrogen) according to manufacturer instructions. PCR amplifications were achieved using miRNA- specific primers (Supplementary Table S1) and NCode SYBR miRNA qRT-PCR kit (Invitrogen). Relative mRNA and miRNA expression was calculated using the ΔΔCt method [25] and normalized using expression of 3 housekeeping genes (ribosomal protein L27a (RPL27a), 18S, and b-actin) for mRNAs, and U6 small nuclear RNA (U6) for miRNAs.

Vector construction

For luciferase assays, the 3'-untranslated region (UTR) of gilthead seabream BMP-2 transcript was inserted into XbaI site of pGL3-Control vector (Promega) downstream of firefly luciferase (F-Luc) coding sequence. 3'UTR was amplified from Marathon cDNA cDNA libraries (Clontech) using gene-specific primers (Supplementary Table S1) and Klen Taq Polymerase mix (Clontech). Mutations in polyadenylation signal and miR-20a binding sites were achieved using 50 ng of pGL3–3'UTR constructs, specific primers containing point mutations (designed according to manufacturer instructions; Supplementary Table S1) and the QuickChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies). PCR reaction was treated with DpnI restriction endonuclease to cut methylated template DNA and used to transform

XL10-Gold cells (Agilent Technologies) according to manufacturer instructions. For miR-20a overexpression, oligonucleotides containing forward and reverse sequences of zebrafish pre-miR-20a (Supplementary Table S1) were annealed then inserted into pcDNA6.2-GW/EmGFP-miR vector downstream of GFP coding sequence using the BLOCK-iT Pol II miR RNAi Expression Vector kit (Invitrogen). The BMP-responsive luciferase reporter vector (BRE-Luc) was kindly provided by Dr. Peter ten Dijke (Leiden University Medical Center, Leiden, The Netherlands) [26].

Luciferase assays

HEK 293 cells were seeded in 24-well plates at 8 x 10^4 cells/well, further cultured for 14–16 h and transfected with 5 ng of the pGL3–3'UTR construct and 12.5 ng of pRL-TK vector (Promega) carrying renilla luciferase gene (R-Luc) using 1.5 ll of X-treme- GENE HP transfection reagent (Roche). When appropriate, 5 ng of pcDNA6.2-miR20a vector was co-transfected in HEK 293 cells. ABSa15 cells were seeded in 12-well plates at 8 x 10^4 cells/well, further cultured for 14–16 h and transfected with 600 ng of BRE- Luc vector and 600 ng of pRL-SV40 vector using 1.5 ll of FuGene HD (Roche). After 48 h, transfected cells were lysed and luciferase activities were measured using Dual-Luciferase Reporter Assay system (Promega). Relative luciferase activity was determined from the ratio F-Luc/R-Luc.

Establishment of cell clones overexpressing miR-20a

ABSa15 cells were seeded in 6-well plates at 2 x 10^5 cells/well, further cultured for 14–16 h and transfected with 2.4 μg of pcDNA6.2-miR20a vector using 3 μl of FuGeneHD (Roche). After 24 h, cells were sub-cultured into a 10-cm culture dish containing DMEM supplemented with 2 μg/ml of blasticidin (determined as described in the manual of BLOCK-iT Pol II miR RNAi Expression Vector kit). After approximately 30 days in selective medium (renewed once a week), cell colonies expressing GFP were identified using Olympus IX-81 fluorescence microscope and sequentially sub-cultured into 24-well, 6-well and 10-cm culture dishes.

Protein extraction and western-blot analysis

Proteins were extracted from cell cultures using lysis buffer containing Tris (50 mM), sodium chloride (150 mM), NP-40 (1% m/v), glycerol (10% v/v), magnesiumchloride (10 mM), sodium orthovanadate (10 mM) and protease inhibitor cocktail (cOmplete, Roche). Protein concentrations were determined using the Bradford protein assay (Bio-Rad). Proteins were fractioned using 4–12% acrylamide NuPAGE Novex Bis-Tris gels (Invitrogen) and transferred onto PVDF membranes (Millipore) using the XCell SureLock Blot module (Invitrogen). The following antibodies were used for western-blot: anti-zebrafish BMP-2b rabbit IgG conjugate (AnaSpec; 1:500 dilution), anti-avian b-Actin mouse IgG conjugate (Santa Cruz Biotechnology; 1:500 dilution), anti-rabbit IgG peroxidase conjugate (Sigma–Aldrich; 1:30,000 dilution) and anti-mouse IgG-peroxidase conjugate (Sigma–Aldrich; 1:30,000 dilution). Chemiluminescent signals were detected using the Western Lightning ECL kit (Perkin Elmer) and Hyperfilm ECL (Amersham, GE Healthcare) then quantified through densitometry analysis.

Results and discussion

Expression of miR-20a is up-regulated during in vitro mineralization in fish

Levels of miR-20a expression and ECM mineralization were determined by qPCR and von Kossa staining, respectively, in confluent cultures of gilthead seabream ABSa15 cells exposed to mineralogenic cocktail for 4 weeks or left untreated (Fig. 1). While expression of miR-20a remained basal and constant in control non-mineralizing cultures, it was strongly up-regulated (up to 6- folds) during the first 2 weeks cultured in mineralogenic medium, a period corresponding to the onset of in vitro mineralization, then remained constant as ECM mineralization progressed in the following 2 weeks, suggesting a role for miR-20a in mechanisms of cell differentiation towards a phenotype of ECM mineralization.

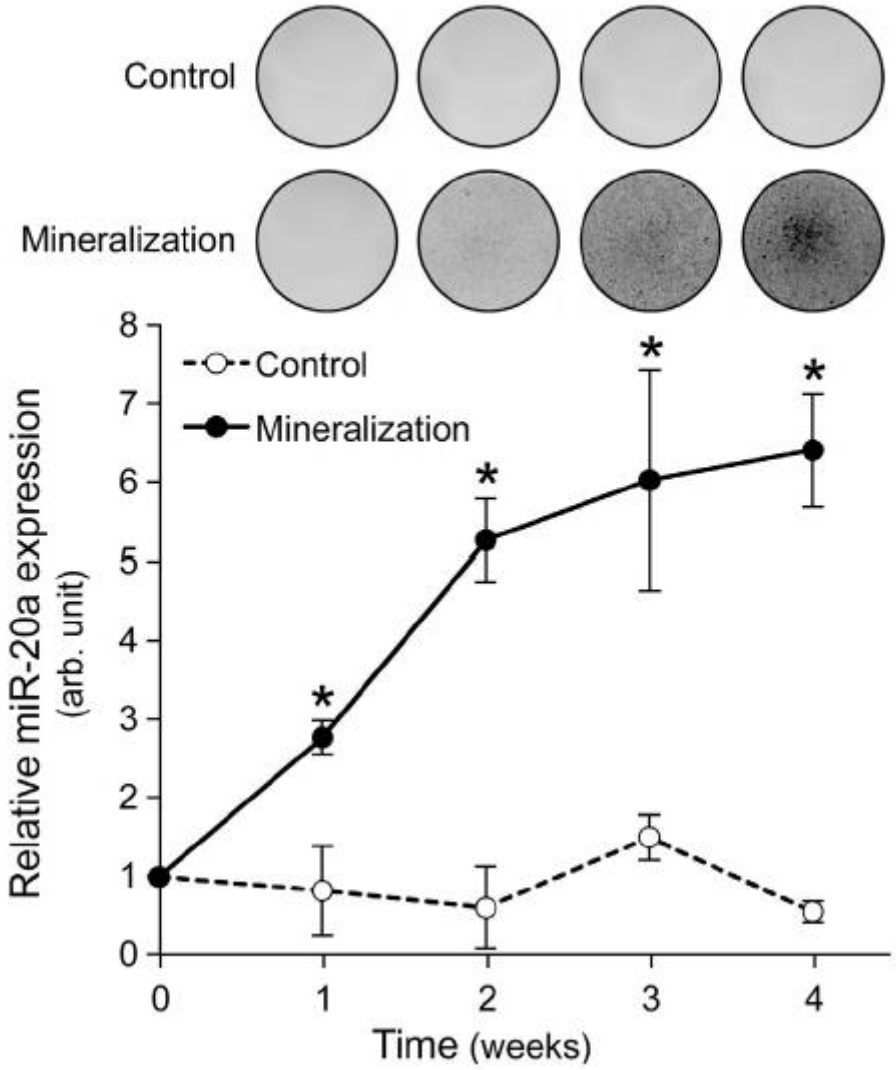

Fig. 1. Relative expression of miR-20a in mineralizing gilthead seabream ABSa15 cells. Extracellular matrix mineralization was induced in confluent cultures by supplementing medium with 50 μg/ml of L-ascorbic acid, 10mM b-glycerophosphate and 4mMCaCl2. Control cultures were left untreated. Expression of miR-20a was determined by qPCR and normalized using U6 small RNA expression. Representative pictures of von Kossa-stained cultures are presented above qPCR data. Asterisks(*) indicate values statistically different from respective control at specific specific time of mineralization (n≥3; Student's t-test, $p < 0.01$).

Overexpression of miR-20a decreases ECM mineralization and the activity of BMP canonical pathway

To further study this role, clones of ABSa15 cells overexpressing miR-20a were developed through the stable transfection of pcDNA6.2-GW/EmGFP-miR-20a construction. Three clones, homogeneously expressing GFP and therefore miR-20a (data not shown), were isolated and overexpression of miR-

20a was confirmed by qPCR (6.3, 11.9 and 10.9-fold increase in clones 1, 2 and 3, respectively; Fig. 2A). Overexpressing (OE) clones were exposed to mineralogenic cocktail for 4 weeks and mineral deposition was evaluated once a week. Onset of ECM mineralization occurred after 2 weeks of exposure; at that time mineral deposition was significantly reduced by 44, 48 and 51% in clones 1, 2 and 3, respectively (Fig. 2B). Comparative analysis of these results with those presented in Fig. 1 suggests that miR-20a could have a specific role in early cell differentiation, i.e., from 0 to 2 weeks of mineralogenic treatment, progressively inhibiting this process and allowing for mineralogenic mechanisms to occur. To support this hypothesis, ECM mineralization in cells overexpressing miR-20a was delayed but not impaired. In fact, at later stages of ECM mineralization (3 and 4 weeks), mineral deposition remained lower in OE clones than in wild-type cells, but differences were not as accentuated (results not shown), indicating a partial recovery. It has been recently reported that bone morphogenetic protein (BMP) pathway is targeted by miR-20a [15]; BMP pathway is central to osteogenesis, promoting osteoblast differentiation and ECM mineralization in fish [14,27–29] and mammals[27,30], and its repression would certainly impair ECM mineralization in ABSa15 cell line. To test this hypothesis, wild-type ABSa15 cells and clones OE-1, -2 and -3, were transfected with the BRE-Luc vector, a construct recently developed to investigate the activation of BMP-pathway and where BMP responsive elements (BRE) control the expression of firefly luciferase gene [26]. Luciferase activity was significantly reduced in OE clones (Fig. 2C), indicating that BMP canonical pathway was affected upon overexpression of miR-20a. Expression of miR-20a was also silenced in ABSa15 cells using a construct where a siRNA against miR-20a was cloned into pcDNA6.2-GW/EmGFP vector. Two cell clones displaying reduced miR-20a expression were isolated and treated for mineralization. Surprisingly, no significant changes in ECM mineralization were observed in these clones versus wild-type ABSa15 cells (data not shown), suggesting that compensatory mechanisms may exist.

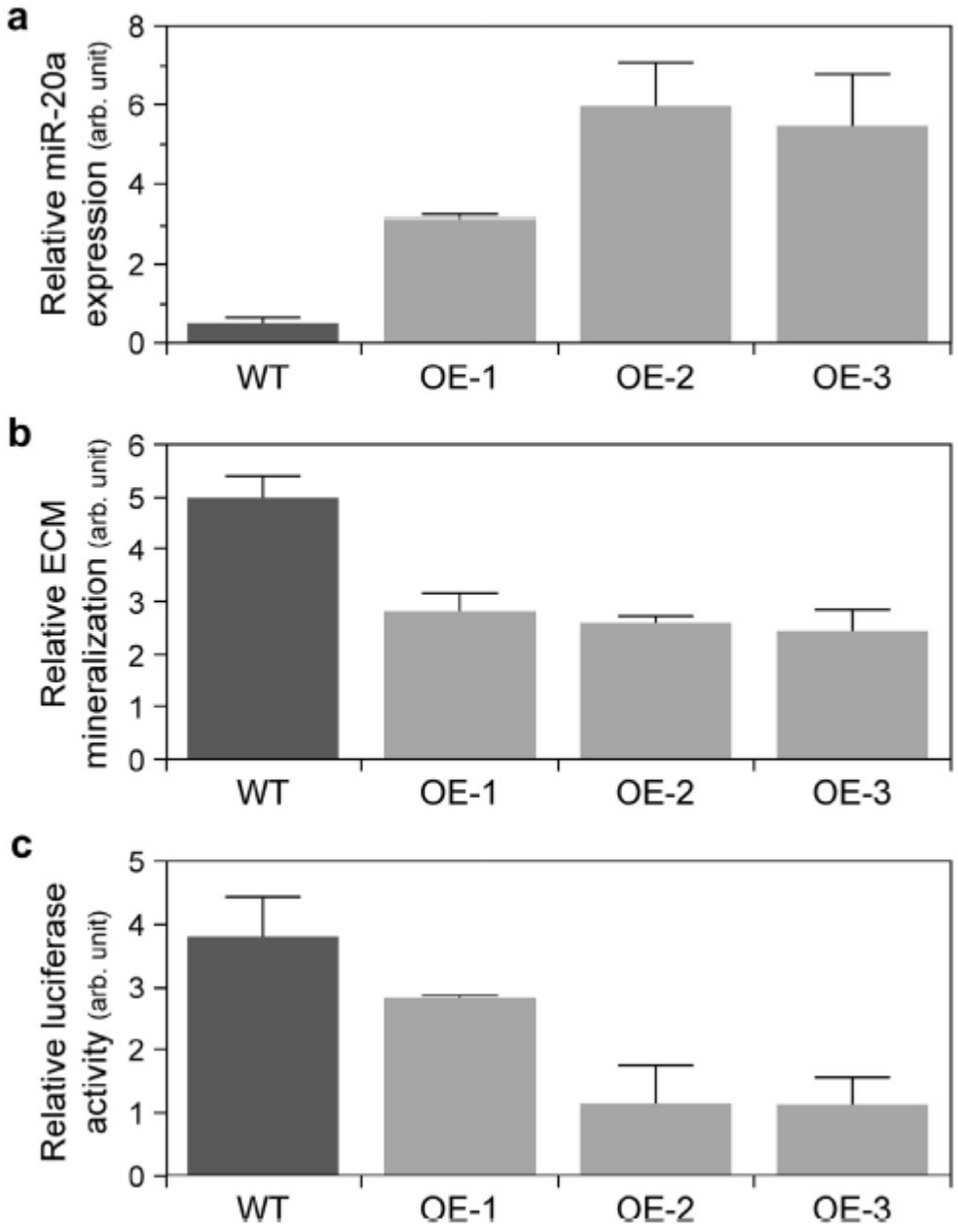

Fig. 2. Effect of miR-20a overexpression in gilthead seabream ABSa15 cells. Relative expression of miR-20a (a) ECM mineralization (b) and reporter gene analysis of the canonical BMP signaling pathway (c) in wild-type cells (WT) and clones overexpressing miR-20a (OE-1, -2 and -3). Expression of miR-20a was determined in confluent cultures by qPCR and normalized using U6 small RNA expression. Mineral deposition was revealed after 2 weeks by von Kossa staining and evaluated by densitometry analysis. Reporter gene analysis was performed in cells transfected with BRE-Luc vector containing BMP-responsive elements upstream of luciferase gene. Relative luciferase activity was calculated as the ratio of firefly and renilla luciferase activities (F-Luc/R-Luc). All values in OE clones were statistically different from values in WT cells ($n \geq 3$; one-way ANOVA, $p < 0.05$).

BMP-2 transcripts contain an evolutionary conserved binding site for miR-20a

To further investigate the anti-mineralogenic action of miR-20a through BMP pathway, the 3'UTR of gilthead seabream BMP-2 transcript was analyzed *in silico* for the presence of miRNA binding sites. A search in GenBank sequence database using on-site blast facilities identified 2 transcript variants different in the length of their 3'UTR (GenBank accession numbers AY500244 and JF261172). A canonical polyadenylation signal (AAUAAA) was identified in both transcripts 16–24 nt upstream of poly(A) tail (Fig. 3A). Approximately 50% of mammalian protein-coding genes have more than one polyadenylation signal and can code for transcripts that differ in their 3'UTR [31]. Since 3'UTRs contain binding sites for proteins that regulate mRNA stability [32] and for miRNAs that regulate mRNA translation [33], alternative polyadenylation has been associated with post-transcriptional regulation of transcripts. Thus the 3'UTR region of the long variant of gilthead seabream BMP-2 transcript was searched for miRNA binding sites using PITA algorithm (genie.weizmann.ac.il) and respective on-site miRNA database. Since PITA database only contained mammalian miRNA sequences, the conservation of predicted miRNAs (from mammals to zebrafish) was assessed using miRBase (http:// www.mirbase.org). A binding site for miR-20a was predicted with a ΔΔG score of -10.48 J/mole (sites with a score below -10 J/mole are likely to be functional if miRNA is endogenously expressed [34]). BMP-2 transcript was further analyzed using TargetScanFish release 6.2 (http://www.targetscan.org/fish_62), an online tool recently developed to search miRNAs binding sites in zebrafish sequences, and the presence of a miR-20a binding site was confirmed in BMP-2 3'UTR. Although TargetScanFish analysis also predicted the presence of binding sites for other members of the miR-17 family, which share similar seed regions, low binding energies were calculated by PITA for these binding sites, suggesting that that they are less likely to bind miRNA binding site than miR- 20a. The conservation of miR-20a binding site in BMP-2 transcripts throughout vertebrate evolution, a critical feature in miRNA binding predictions [35,36], was investigated using BMP-2-related sequences available in GenBank database.

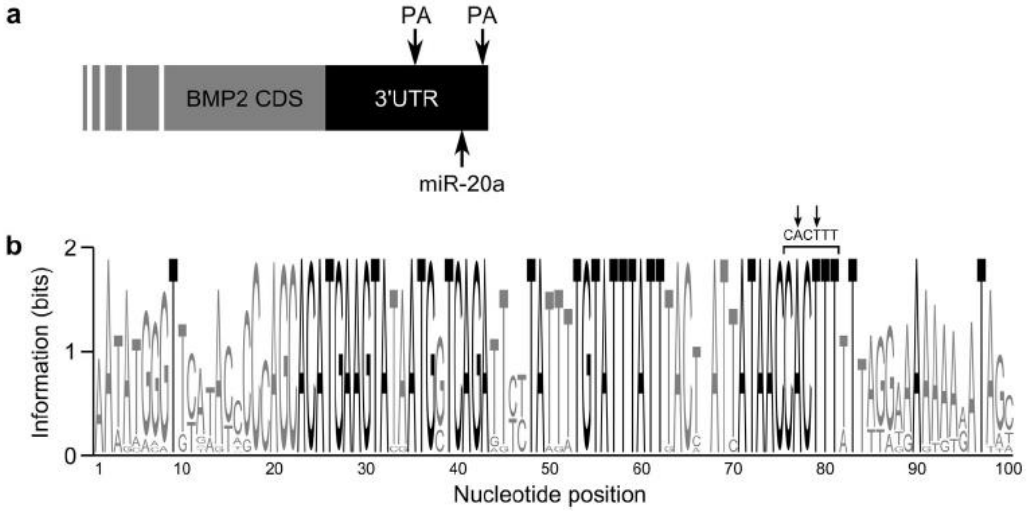

Fig. 3. Prediction of miR-20a binding sites in the 30 untranslated region (UTR) of BMP-2 transcripts using PITA algorithm and TargetScanFish release 6.2. (a) schematic representation of the 3'UTR of gilthead seabream BMP-2 transcript where polyadenylation signals (PA) and miR-20a binding site are indicated. (b) Sequence logos of the 30 untranslated region of mammalian, sauropsidian, amphibian and fish BMP-2 transcripts; miR-20a seed region (CACTTT) is indicated on top of the logo and arrows indicate nucleotides mutated for functional analysis of miR-20a binding; overall height of each letter corresponds to level of nucleotide conservation among species at that position; black letters indicate 100% conservation.

Nineteen 3'UTR sequences of mammalian (10), sauropsidian (2), amphibian (3) and bony fish (4) BMP-2 transcripts were collected, aligned using ClustalW (align.genome.jp; Supplementary Fig. S2), then displayed as sequence logos using Weblogo (weblogo.berkeley.edu). A remarkable conservation of putative miR-20a binding site, in particular the seed region, was observed (Fig. 3B), further evidencing the probable post-transcriptional regulation of BMP-2 transcript by miR-20a. A second seed region for miRNAs of the miR-17 family (including miR-20a) was identified in the 3'UTR of mammalian, birds and amphibians BMP-2 transcripts; it was however absent in fish BMP-2 transcripts. Although its ΔΔG score was low (-6.76 J/mole in human sequence), which may indicate a false positive, future studies should aim at determining whether any miRNA of the miR-17/92 cluster, in particular miR-20a, bind to this tetrapod-specific site and whether post-transcriptional regulation of BMP-2 transcripts has evolved throughout vertebrate evolution towards a tighter control by miR-17 family. Following the report by Brock et al. [15] evidencing the presence of a binding site for miR-20a in the 3UTR of human BMPR2 transcript, GenBank database was searched for vertebrate BMPR2-related sequences. Fourteen

sequences were collected (mammals (8), sauropsids (3), and bony fish (3)) and aligned using ClustalW. A remarkable conservation of miR-20a binding site was observed, in particular the seed region (Supplementary Fig. S3). Although both PITA and TargetScanFish returned low scores for the binding of miR-20a to the miRNA site identified in zebrafish BMPR2 transcript, the remarkable conservation of the seed region suggests that this site could be functional in fish. Although this remains to be demonstrated, we propose that BMP signaling pathway and downstream processes are regulated by miR-20a through its action on BMP-2 but also on BMPR2 transcripts.´

Seabream BMP-2 transcript is post-transcriptionally regulated by miR- 20a

In silico prediction of miRNAs binding sites in BMP-2 3'UTR sequences clearly indicated miR-20a as a strong candidate for the post-transcriptional regulation BMP-2 transcript. To validate this hypothesis, firefly luciferase activity was measured in extracts of HEK 293 cells transfected with reporter vector carrying 3'UTR region downstream of luciferase gene and, when appropriate, with expression vector carrying miR-20a downstream of CMV promoter (Fig. 4). Polyadenylation signal upstream of miR-20a binding site was mutated to avoid premature termination of the fusion transcript. Since miR-20a expression was recently reported in HEK 293 cells [37], possible suppressive effects of endogenous miR- 20a in control conditions were considered in these experiments. While luciferase activity of BMP-2 construction was slightly reduced (19%) upon miR-20a overexpression, it was up-regulated (62%) upon mutation of the binding site, suggesting a pre-existing repression by endogenous miR-20a and therefore a post-transcriptional regulation of BMP-2 through miR-20a binding site. Although a slight decrease of luciferase activity was observed upon overexpression of miR-20a, we could not exclude that other members of the miR-17 family, which share the same binding sites with miR-20a and are expressed at similar levels in HEK 293 cells [37], may also regulate seabream BMP-2 transcript. However, as stated previously, according to PITA analysis binding of other members of miR-17 family to BMP-2 transcript is less probable due to their association to lower binding energies. Nevertheless, to

better understand the specific effect of miR-20a, protein and transcript levels of gilthead seabream BMP-2 were determined in OE clones. In all OE clones, overexpression of miR-20a resulted in a slight reduction of BMP-2 production at 0 weeks (from 18% to 46%; Fig. 5A) and in a strong reduction after 2 weeks (from 80% to 100%; Fig. 5A). A strong reduction (from 60% to 80%) of BMP-2 transcript levels (long transcript) was also observed after 2 weeks in all OE clones (Fig. 5B). These data further demonstrated the regulation of BMP-2 by miR-20a and also suggested that miR-20a action on BMP-2 is probably related to mRNA degradation. Due to the lack of a suitable antibody to detect fish BMPR2, the action of miR- 20a on this protein in fish is still unknown. Furthermore, qPCR analysis of BMPR2 transcripts in WT ABSa15 cells and OE clones did not reveal any significant changes. Data collected in mammalian systems pointed towards the inhibition of protein translation Brock et al. [15], but whether this mechanism applies also in fish remains to be confirmed.

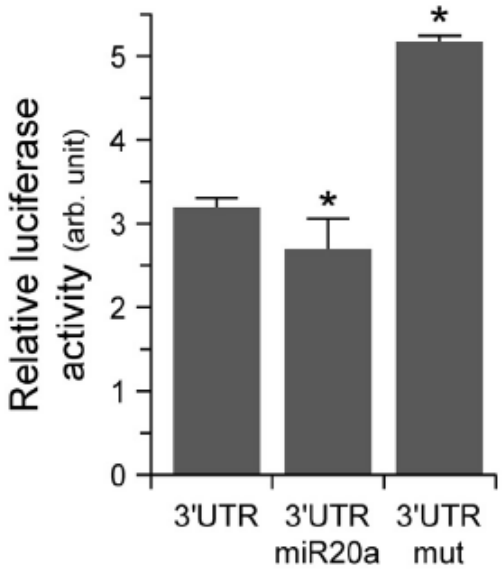

Fig. 4. Interaction between miR-20a and the 3'UTR of gilthead seabream BMP-2 (SauBMP-2) transcript. HEK 293 cells were transfected with pGL3 vector carrying the 3'UTR of SauBMP-2 transcript (3'UTR) or the 3'UTR mutated for miR-20a binding sites (3'UTR mut) downstream of luciferase gene. HEK 293 cells were also co-transfected with 3'UTR constructs and pcDNA6.2 carrying miR-20a (3'UTR miR20a) downstream of CMV promoter. Relative luciferase activity was calculated as the ratio of firefly and renilla luciferase activities. Asterisks (*) indicate values statistically different from respective 3'UTR value ($n \geq 3$; one-way ANOVA, $p < 0.05$).

The role of miR-20a in bone formation is far from being understood. On one hand, the nature of known targets of miR-20a, either identified through this study – BMP-2 – or identified in a previous study – BMPR2 [15] – suggest that miR-20a can repress bone cell differentiation and ECM mineralization [30]. On the other hand, miR-20a repression of MAPK [38], a pathway that was shown to inhibit bone cell differentiation in mammals and fish [27–29], suggests that miR-20a could also promote bone cell differentiation in fish. Accordingly, miR-20a was recently shown to induce osteogenic differentiation in human mesenchymal stem cells (hMSC) through repression of antagonists of BMP pathway Bambi, Crim1 and PPAR-c [12]. In contrast, the role of miR-20a in bone formation was further investigated in vivo in a study using mouse knockout models for the miR-17/92, a cluster of miRNAs in which miR-20a is included [39]. While the development of homozygotic miR-17/ 92 knockouts is severely compromised due to lethal cardiac and lung defects, heterozygous models showed significantly reduced trabecular and cortical bone formation, and impaired osteoblast differentiation [40]. Data collected within the scope of this work is in favor of miR-20a inhibiting bone cell differentiation/mineralization through the repression of BMP pathway. This discrepancy could be related to distinct regulatory mechanisms in different cell systems: ABSa15 is a skeletal cell line established from calcified cartilage of branchial arches of a teleost fish; it displays gene expression patterns resembling those of chondrocyte-like cell types, including: (i) mild up-regulation of TNAP (tissue non-specific bone-related alkaline phosphatase), COL1A1 (type I collagen a1), SPARC (secreted protein acidic cysteine-rich; also known as osteonectin) and SOX9a (SRY-box containing gene 9a) earlier in differentiation and down-regulation later during mineralization; (ii) strong up-regulation of MGP (matrix Gla protein) and SPP1 (secreted phosphoprotein 1; also known as osteopontin) from non-differentiated to mineralized cells; and (iii) absence of osteocalcin expression in all stages (Supplementary Fig. S4). Supporting the dual effect of miR-20a, a recent study showed that miR-17, a miRNA that belongs to miR-17/92 cluster and shares the same ‘‘seed’’ and predicted targets with miR-20a, could either inhibit or promote osteogenic differentiation in human periodontal ligament

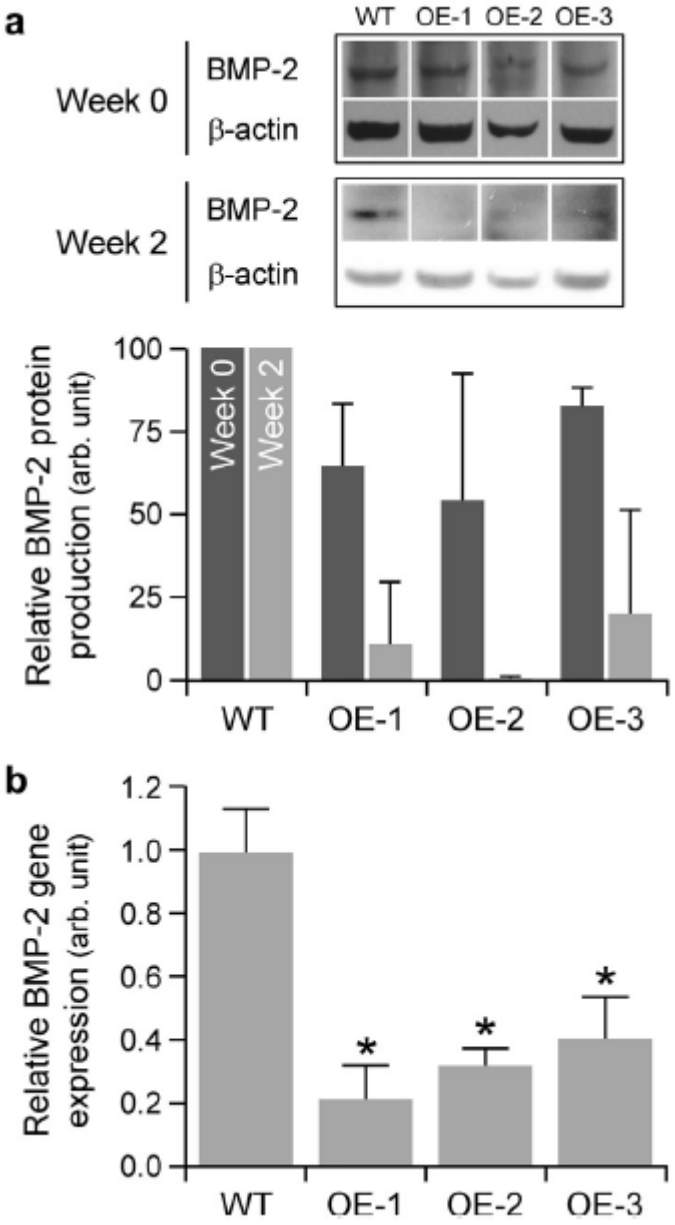

Fig. 5. Levels of BMP-2 protein production (a) and gene expression (b) in wild-type ABSa15 cells (WT) and clones overexpressing miR-20a (OE-1, -2 and -3). Production of BMP-2 protein was determined in cell cultures at time 0 and after 2 weeks of mineralization by densitometry analysis of western-blot signals and normalized using b-actin signals. Expression of BMP-2 gene was determined by qPCR and normalized using RPL27a housekeeping gene expression (n.b. similar expression data were collected using 18S and b-actin housekeeping genes; data not shown). Asterisks (*) indicate values statistically different from WT (n≥3; one-way ANOVA, $p<0.05$).

tissue stem cells depending on whether these where collected from healthy donors or patients suffering from inflammatory process, respectively [41]. Interestingly, this opposite effect was associated with the differential expression of Smad ubiquitin regulatory factor one (Smurf1), a regulator of BMP pathway

and a direct target of miR-17. In another study, Brock and colleagues evidenced in human and mouse the post-transcriptional regulation of the cell surface receptor BMPR2 by miR-20a and proposed that the up- regulation of miRNA expression may be a key feature in the development of pulmonary arterial hypertension (PAH), through the action of BMP signaling pathway on endothelial and smooth muscle cell differentiation and matrix formation [15,16]. These authors showed that antagonizing miR-20a using an antagomiR restored BMPR2 mRNA/protein levels and a functional BMP signaling in a mouse model of hypoxia-induced PAH [16]. Consequently disease development was reduced and pulmonary arterial haemodynamics were improved in antagomiR-20a treated animals [16]. Smad5, an intermediate of BMP signaling pathway, was also proposed to be targeted by miR-20a but this hypothesis was never confirmed either by luciferase reporter assays or western-blot analysis [16]. Interestingly, miR-17/92 cluster, in particular miR- 20a, has been shown to repress type II transforming growth factor b receptor (TGFBR2) [42], which is involved in osteogenesis (and chondrogenesis) through the action of TGFb signaling on osteoblast recruitment and proliferation, and matrix formation [43]. Since BMPR2 and TGFBR2 belong to the same cell surface receptor family and activate similar transduction pathways, it will be interesting to address in future studies the role of their post-transcriptional regulation by miR-20a during osteogenesis (and in a more general manner during skeletogenesis) and whether a deregulation of this mechanism could lead to bone/skeletal diseases.

Overexpression of miR-20a up-regulates the expression of the matrix Gla protein, a calcification inhibitor

In order to better understand the underlying mechanisms of miR-20a inhibitory role on ECM mineralization of ABSa15 cells, expression of several markers of bone cell differentiation/mineralization was investigated in wild-type cells and in clones overexpressing miR-20a. Initially, the expression of each bone-related marker was investigated during ECM mineralization of ABSa15 cells, and as mentioned before, this analysis suggested a possible association of ABSa15 cells to a chondrocytic lineage (Supplementary Fig. S4). After 2

weeks of treatment, when overexpression of miR-20a strongly inhibited mineral deposition, expression levels of TNAP, COL1A1 and MGP were significantly up-regulated in all three OE clones, ranging between 3.7–6.4-fold, 3.1–5.5-fold and 2.1–5.1-fold, respectively (Fig. 6), while other bone-related genes did not reach significant differences or remained undetected (data not shown). The strong up-regulation of MGP, a well-known inhibitor of arterial calcification in mammals [44] which has been also associated with ECM mineralization in fish [23,45–47], could explain miR-20a inhibitory effects in ABSa15 cell mineralization. In mammalian systems, MGP was demonstrated to bind BMP-2 and prevent its association with BMPR2, which is necessary for the activation of BMP pathway and consequent stimulation of bone formation [48,49]. Furthermore, in calcifying vascular cells this mechanism involved a feedback control regulation, where MGP expression levels appear to be negatively correlated with BMP-2 availability [50]. Therefore, an up-regulation of MGP in ABSa15 OE clones is likely to increase its binding to BMP-2 and thus contribute to block BMP pathway, enhancing the effect of post-transcriptional regulation of BMP-2 by miR-20a. Regarding TNAP and COL1A1, these are promoters of ECM formation and mineralization [51] and it is therefore difficult to explain the anti-mineralogenic effect of miR-20a through their up-regulation. Since data available on literature regarding regulation of TNAP and COL1A1 by miR-17/ 92 is still contradictory [40], this effect should be addressed in future studies.

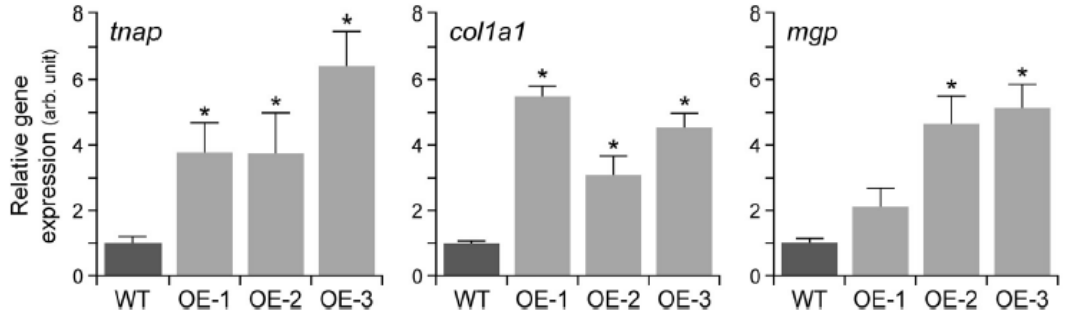

Fig. 6. Levels of alkaline phosphatase (TNAP), type I collagen a1 (COL1A1) and matrix Gla protein (MGP) gene expression in mineralizing wild-type ABSa15 cells (WT) and clones overexpressing miR-20a (OE-1, -2 and -3). Gene expression was determined after 2 weeks of mineralization by qPCR and normalized with RPL27a housekeeping gene expression (n.b. similar expression data were collected using 18S and b-actin housekeeping genes; data not shown). Asterisks (*) indicate values statistically different fromWT (n≥3; one-way ANOVA, $p < 0.05$).

Conclusions

We present here novel data (i) describing the up-regulation of miR-20a during ECM mineralization of a fish mineralogenic cell line (ABSa15), (ii) evidencing the anti-mineralogenic effect of miR-20a in this cell line, (iii) showing inhibition of the BMP pathway by miR-20a, (iv) identifying binding sites for miR-20a in the 3'UTR of gilthead seabream BMP-2 and zebrafish BMPR2 transcripts that were conserved in vertebrate and (v) demonstrating the post-transcriptional regulation of BMP-2 by miR-20a (binding site in BMPR2 transcript may also be active but this remains to be demonstrated). We propose that low levels of expression of miR-20a in undifferentiated cells may account for a higher activity of BMP signaling and consequent osteogenic differentiation. Then, in the course of ECM mineralization, miR-20a becomes more expressed to inhibit this process through BMP-2 (and possibly also BMPR2) regulation (Fig. 7). Alternative mechanisms of action, such as activation of MAPK pathway either directly by miR-20a or indirectly through non-canonical BMP pathway, cannot be excluded but remain to be demonstrated in ABSa15 cells. Furthermore, effect on MGP suggests that this protein is likely to play a role in the inhibitory mechanism observed. Results obtained from previous studies combined with data hereby demonstrated, suggest that miR-20a preferentially targets BMP pathway to promote (hMSC) or inhibit (ABSa15 cells) osteogenic differentiation.

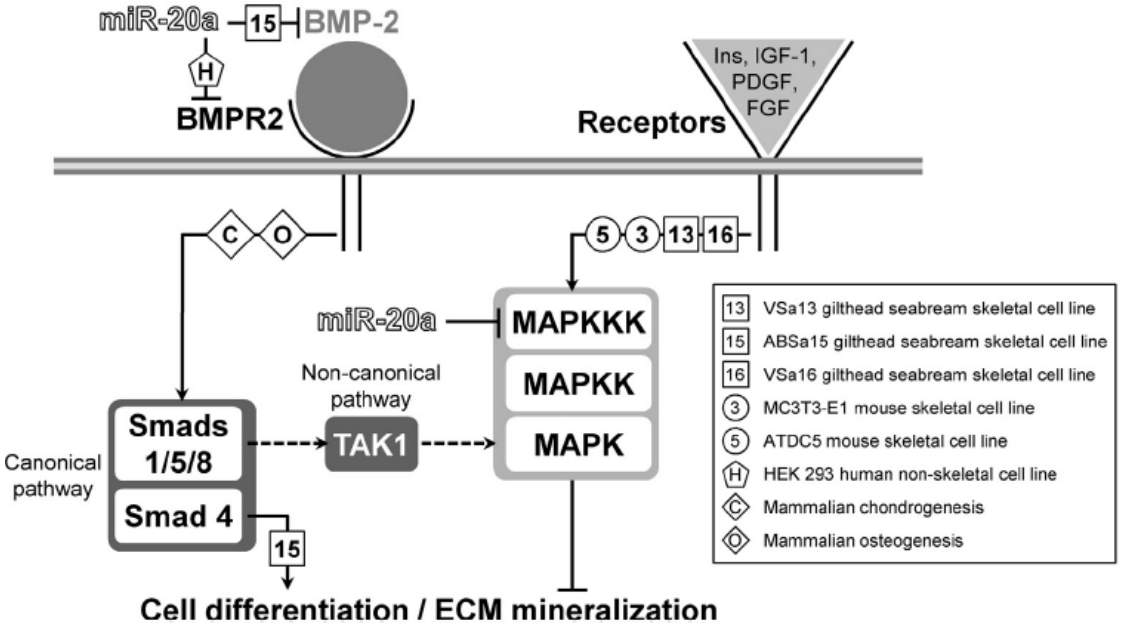

Fig. 7. Putative mechanisms of action for miR-20a anti-mineralogenic effect. Arrows and intersected lines indicate activation and repression, respectively. Solid arrows indicate pathways likely to be activated during differentiation of ABSa15 cells. Dashed arrows indicate pathways most probably not activated in ABSa15 cells. Smads 1, 5, 8 and 4 are intermediates of the BMP canonical pathway. TAK1 (TGF-b-activated kinase 1) is an intermediate of the BMP non-canonical pathway. MAPK, MAPKK and MAPKKK are intermediates of the mitogen-activated protein kinase (MAPK) pathway.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.abb.2013.12.009.

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