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(54) **Title**: ANTISENSE RNA TARGETING CXCR4

(57) **Abstract**: MiR-146a and antagomirs thereof are useful in controlling expression of CXCR4 and megakaryopoiesis, tumor metastasis, HTV entry and/or Rheumatoid Arthritis.

Antisense RNA Targeting CXCR4

The present invention relates to the use of antisense RNA.

MicroRNAs (miRs) are a class of regulatory, single-stranded RNAs of about 22 nucleotides (Bartel, 2004; He and Hannon, 2004; Ambros, 2004). MiRs repress protein expression at post-transcriptional level, mostly through base pairing to the 3' untranslated region (UTR) of the target mRNA, thus leading to its reduced translation and/or degradation (Valencia-Sanchez et al., 2006). MiRs control basic biological functions, such as cell proliferation and differentiation (Zhao et al., 2005; Naguibneva et al., 2006; Chen et al., 2006). Furthermore, in diverse types of cancer miRs may function as "oncomirs" (Esquela-Kerscher and Slack, 2006). Despite these advances, only few targets for the >500 known mammalian miRs have been validated so far. Particularly, relatively little is known on the role of miRs in hematopoietic differentiation (Chen et al., 2004; Fazi et al., 2005; Felli et al., 2005; Fontana et al., 2007).

The promyelocytic leukemia zinc finger protein, PLZF, is a transcription factor involved in the regulation of development (Kelly and Daniel, 2006), hematopoietic proliferation and differentiation (Ball et al., 1999; Labbaye et al., 2002), leukemogenesis (Melnick and Licht, 1999; Parrado et al., 2000) and tumorigenesis (Felicetti et al., 2004). However, the molecular mechanisms through which PLZF exerts its regulatory effects on hematopoiesis are still unknown,

The chemokine receptor 4, CXCR4, and its ligand SDF-1, are key molecules in the process of homing/mobilization of normal hematopoietic and leukemic cells (Peled et al., 1999; Gazitt, 2004; Lapidot et al., 2005; Ratajczak et al., 2006), particularly at stem cells level (Kucia et al., 2005; Burger and Kipps 2006). SDF-1 acts as a mobilization or retention signal in hematopoiesis (Cottler-Fox et al., 2003; Ruiz de Almodovar et al., 2006), while CXCR4 regulation by cytokines is an important step in myeloid cell mobilization (Kim et al., 2006). CXCR4 acts as a transporter for internalization and secretion of SDF-1 across the bone marrow-blood barrier (Dar et al., 2005). Inhibition of CXCR4 blocks thrombopoiesis in vivo (Avecilla et al., 2004), indicating that the integrity of the SDF-1/CXCR4 pathway is indispensable for megakaryocyte proliferation and differentiation/maturation (Pang et al.,

2005). Nevertheless, the mechanism controlling CXCR4 expression in angiogenesis and hematopoiesis is not fully understood.

Surprisingly, we have discovered that miR-146a targets CXCR4 mRNA. Accordingly, by up-modulating miR146a, CXCR4 can be inhibited. Conversely, by down-modulating miR-146a, CXCR4 inhibition can be lifted and upregulation of CXCR4 stimulates megakaryopoiesis.

Analysis of the sequence of the CXCR4 gene promoter did not reveal any functional PLZF binding site, suggesting that CXCR4 is not a direct target gene of PLZF. However, we discovered a discrepancy between stable CXCR4 mRNA levels and increased protein levels in K562-PLZF cells. Since miR-146 is sharply downmodulated in K562-PLZF cells, we formed a hypothesis that CXCR4 mRNA may be targeted by miR-146a. To confirm this, we used a range of different bioinformatic algorithms. However, only a single algorithm supported this hypothesis. Despite this setback, we conducted further experiments and surprisingly found that miR-146a does in fact target CXCR4 mRNA *in vivo*.

Furthermore, we have also surprisingly found that that PLZF represses miR-146a transcription. In hematopoietic progenitor cell (HPC) culture undergoing unilineage megakaryocytic (Mk) differentiation and maturation, the increasing expression of PLZF inhibits miR-146a expression, leading to enhanced CXCR4 protein levels. Functional studies showed that miR-146a or PLZF siRNA transfection similarly downmodulate CXCR4 translation and impair proliferation, differentiation and maturation of Mk cells, indicating that the PLZF/miR-146a/CXCR4 cascade controls normal megakaryopoiesis.

Thus, in a first aspect, the present invention provides use of antisense RNA specific for all or part of the 3' untranslated region of CXCR4 protein mRNA in therapy.

Up-modulating miR-146a (down-modulation of CXCR4) may be achieved, for instance, by administering or increasing expression of miR-146a, for instance administering it directly or in the form of a vector with a coding sequence for miR-146a preferably under the control of a suitable promoter, or by reducing antagomir (anti-miR 146a) levels. It may also preferably be achieved by decreasing the levels or expression of PLZF, for instance by

increasing the levels of inhibitory antisense RNA targeted to PLZF. Down-modulation of CXCR4 leads to reduced megakaryopoiesis.

Conversely, down-modulating miR-146a (up-modulation of CXCR4) may be achieved, for instance, by increasing antagomir (anti- miR-146a) levels, increasing PLZF levels, for instance using a vector with a coding sequence for anti-miR-146a and/or PLZF, preferably under the control of a suitable promoter. It may also preferably be achieved by reducing the levels of inhibitory antisense RNA targeted to PLZF. Up-modulation of CXCR4 leads to increased megakaryopoiesis.

Preferably, therapy is treatment of cancer, preferably tumour metastasis. Preferably, therapy is treatment of HIV, most preferably by prevention of HIV entry into host cells. It is also preferred that the therapy is treatment of Rheumatoid Arthritis. Preferably, the therapy is treatment of other CXCR4-dependent tumours or disease conditions, such as leukaemia and solid tumours.

It is also preferred that the therapy is treatment of the therapy is the modulation of Megakaryopoiesis. By using antisense RNA, such as miR-146a, Megakaryopoiesis can be inhibited. Conversely, use of sense RNA, such as an antagomir can stimulate Megakaryopoiesis.

Preferably, the therapy is via CXCR4 receptor down-modulation or inhibition, particularly by posttranscriptional control of CXCR4 by miR-146a.

It is preferred that the antisense RNA is a micro RNA. Preferably, the antisense RNA has at least 60% homology with a selected region of the 3' untranslated region of CXCR4 protein mRNA. Preferably, the antisense RNA is between about 12 bases and 45 bases in length.

The antisense RNA is preferably a sequence having the same sequence as mature miR-146a, which is preferably the RNA sequence of SEQ ID NO 1 (mature miR-146a 5'-ugagaacugaauuccauggguu-3').

Where a sequence capable of inhibiting miR-146a is required (anti- miR-146a, antagomirs), its sequence is preferably the RNA sequence of SEQ ID NO 2 (anti-sense miR-146a 5'-aacccatggaattcagttctca-3').

The nucleotide sequence of human CXCR4 (SEQ ID NOs. 3 and 4) and PLZF (SEQ ID NO. 5) are given below:

SEQ ID NO.3 (CXCR4)

1 5'-ttttttttct tccctctagt gggcggggca gaggagttag ccaagatgtg actttgaaac
61 cctcagcgtc tcagtgccct tttgttctaa acaaagaatt ttgtaattgg ttctaccaaa
121 gaaggatata atgaagtcac tatgggaaaa gatggggagg agagttgtag gattctacat
181 taattctctt gtgcccttag cccactactt cagaatttcc tgaagaaagc aagcctgaat
241 tggtttttta aattgcttta aaaatttttt ttaactgggt taatgcttgc tgaattggaa
301 **gtgaatgtcc attcctttgc ctcttttgca gatatacact tcagataact acaccgagga**
361 aatgggctca ggggactatg actccatgaa ggaaccctgt ttccgtgaag aaaatgctaa
421 tttcaataaa atcttcctgc ccaccatcta ctccatcatc ttcttaactg gcattgtggg
481 caatggattg gtcatcctgg tcatgggtta ccagaagaaa ctgagaagca tgacggacaa
541 gtacaggctg cacctgtcag tggccgacct cctctttgtc atcacgcttc ccttctgggc
601 agttgatgcc gtggcaaact ggtactttgg gaacttccta tgcaaggcag tccatgtcat
661 ctacacagtc aacctctaca gcagtgtcct catcctggcc ttcatcagtc tggaccgcta
721 cctggccatc gtccacgcca ccaacagtca gaggccaagg aagctgttgg ctgaaaaggt
781 ggtctatgtt ggcgtctgga tccctgccct cctgctgact attcccgact tcatctttgc
841 caacgtcagt gaggcagatg acagatatat ctgtgaccgc ttctacccca atgacttgtg
901 ggtggttgtg ttccagtttc agcacatcat ggttggcctt atcctgcctg gtattgtcat
961 cctgtcctgc tattgcatta tcatctccaa gctgtcacac tccaagggcc accagaagcg
1021 caaggccctc aagaccacag tcatcctcat cctggctttc ttcgcctgtt ggctgcctta
1081 ctacattggg atcagcatcg actccttcat cctcctggaa atcatcaagc aagggtgtga
1141 gtttgagaac actgtgcaca agtggatttc catcaccgag gccctagctt tcttccactg
1201 ttgtctgaac cccatcctct atgctttcct tggagccaaa tttaaaacct ctgcccagca
1261 cgcactcacc tctgtgagca gagggtccag cctcaagatc ctctccaaag gaaagcgagg
1321 tggacattca tctgtttcca ctgagtctga gtcttcaagt tttcactcca gctaacacag
1381 atgtaaaaga ctttttttta tacgataaat aacttttttt taagttacac atttttcaga
1441 tataaaagac tgaccaatat tgtacagttt ttattgcttg ttggattttt gtcttgtgtt
1501 tctttagttt ttgtgaagtt taattgactt atttatataa atttttttg tttcatattg
1561 atgtgtgtct aggcaggacc tgtggccaag ttcttagttg ctgtatgtct cgtggtagga
1621 ctgtagaaaa gggaactgaa cattccagag cgtgtagtga atcacgtaaa gctagaaatg
1681 atccccagct gtttatgcat agataatctc tccattcccg tggaacgttt ttcctgttct
1741 taagacgtga ttttgctgta gaagatggca cttataacca aagcccaaag tggtatagaa
1801 atgctggttt ttcagttttc aggagtgggt tgatttcagc acctacagtg tacagtcttg
1861 tattaagttg ttaataaaag tacatgttaa acttaaaaaa aaaaaaaaaa aa-3'

The preferred coding sequence: 305 ..1375 is shown in bold and underlined above and in the Sequence Listing.

A preferred nucleotide sequence encoding PLZF is shown in SEQ ID NO. 5.

The 3' untranslated (UTR) region of human CXCR4 protein mRNA is provided as SEQ ID NO. 6 :

1376 5'-cacag

1381 atgtaaaaga ctttttttta tacgataaat aacttttttt taagttacac atttttcaga

1441 tataaaagac t**gaccaatat tgtacagttt tta**ttgcttg ttggattttt gtcttgtgtt

1501 tctttagttt ttgtgaagtt taattgactt atttatataa atttttttg tttcatattg

1561 atgtgtgtct aggc**aggacc tgtggccaag ttctta**gttg ctgtatgtct cgtggtagga

1621 ctgtagaaaa gggaactgaa cattccagag cgtgtagtga atcacgtaaa gctagaaatg

1681 atccccagct gtttatgcat agataatctc tccattcccg tggaacgttt ttcctgttct

1741 taagacgtga ttttgctgta gaagatggca cttataacca aagcccaaag tggtataga**a**

1801 **atgctggttt ttcagttttc a**ggagtgggt tgatttcagc acctacagtg tacagtcttg

1861 tattaagttg ttaataaaag tacatgttaa acttaaaaaa aaaaaaaaaa aa-3'

The antisense RNA is preferably specific for all or part of the 3' UTR of CXCR4 protein mRNA. Preferably, it is specific for the full length 3' UTR of CXCR4 mRNA. Preferably, it is specific for at least one, and preferably both, of the following putative miR-146a binding sites: "Site 2" (from 1575 to 1596 bp); and "Site 3" (from 1800 to 1821 bp) as described in Didiano and Hobert, 2006, as shown above in bold and underlined text and also shown in Figure 3c. The binding sites for the present polynucleotide on CXCR4 are preferably one or more of those shown in Figure 3c, especially the highlighted portions thereof and above.

Preferred PLZF binding site sequences shown in Figures 3e and 3g are provided in SEQ ID NOs 7 and 8, where GTAC in the first sequence is mutated to CGGC.

Also provided is an inhibitor or suppressor (antagomir) of miR-146a. Preferably, this is a sense RNA. The invention provides for the use of this inhibitor or suppressor in therapy, preferably in mediation, and preferably stimulation, of megakaryopoiesis.

The sense RNA preferably hybridises to miR-146a under highly stringent conditions, such as 6 x SSC.

Similarly, the invention also provides the use of PLZF to increase CXCR4 expression or reduce miR 146a-mediated inhibition of CXCR4, in therapy, preferably in mediation, and preferably stimulation, of Megakaryopoiesis.

Also provided is a vector comprising the present antisense or antagomir RNA, or DNA encoding said RNA. Preferably, the vector encodes or comprises the mature form of the RNA, where the RNA is a micro RNA. The invention also provides a vector comprising nucleotides encoding PLZF.

The invention also provides a method of treating Cancer, HIV and/or Rheumatoid Arthritis, for example, or other CXCR4-dependent tumours or disease conditions, comprising administering to a patient an antisense RNA, preferably miR-146a, and/or antisense RNA targeted to PLZF, to thereby inhibit CXCR4 expression, as discussed above.

Antisense RNA may be specific for any part of the 3' UTR of CXCR4 protein mRNA, and it will be appreciated that the 3' UTR may vary slightly from individual to individual.

The invention also provides the use of PLZF, or inhibitors thereof, to modulate CXCR4 expression, for instance via miR-146a, and for treatment of conditions associated with CXCR4. In particular, we have also found that siRNA targeted to PLZF, thereby inhibiting or downmodulating PLZF expression, also has a similar effect to upmodualting miR-146a, i.e. inhibition of CXCR4, as well as Mk cell proliferation and differentiation/maturation.

Provides is a method of inhibiting megakaryopoiesis or treating cancer, HIV and/or Rheumatoid Arthritis, or other CXCR4-dependent tumours or disease conditions, comprising administering to a patient an antisense RNA, preferably miR-146a, and/or antisense RNA targeted to PLZF, to thereby inhibit CXCR4 expression.

Alternatively, also provided is a method of stimulating megakaryopoiesis comprising administering to a patient an antagomir of miR-146a and/or PLZF or nucleotides encoding it, to thereby increase CXCR4 expression.

In addition, as noted above, miR need not be 100% faithful to the target, sense sequence. Indeed, where they are 100% faithful, this can lead to cleavage of the target mRNA through the formation of dsRNA. While the formation of dsRNA and cleavage of CXCR4 protein mRNA is included within the scope of the present invention, it is not a requirement that the

antisense RNA be 100% faithful to the target sequence, provided that the antisense RNA is capable of binding the target 3' UTR to inhibit or prevent translation.

Thus, it will be appreciated that the antisense RNA of the present invention need only exhibit as little as 60% or less homology with the target region of the 3' UTR. More preferably, the antisense RNA exhibits greater homology than 60%, such as between 70 and 95%, and more preferably between 80 and 95%, such as around 90% homology. Homology of up to and including 100%, such as between 95 and 100%, is also provided. Suitable methods for assessment of such homology include the BLAST program.

The antisense RNA of the present invention may be as long as the 3' UTR, or even longer. However, it is generally preferred that the antisense RNA is no longer than 50 bases, and it may be a short as 10 bases, for example. More preferably, the antisense RNA of the present invention is between about 12 bases and 45 bases in length, and is more preferably between about 15 and 35 bases in length.

The preferred miR is miR-146a, according to SEQ ID NO. 1. Its mature length is of 22 bases. Thus, a particularly preferred length is between 20 and 25 bases, and especially 22.

The area of the 3' UTR to be targeted may be any that prevents or inhibits translation of the ORF, when associated with an antisense RNA of the invention. The particularly preferred regions are those targeted by miR-146a, and targeting either of these regions with antisense RNA substantially reduces translation of CXCR4 protein.

Regions of the 3' UTR that it is preferred to target include the central region of the 3' UTR and regions between the central region and the ORF. Such regions which are proximal to the ORF are particularly preferred. Other CXCR4 mRNA sequences, such as the coding region for instance, may also be targeted. It is preferred that the antisense RNA of the present invention is a short interfering RNA or a micro RNA.

The present invention further provides mutants and variants of miR-146a. In this respect, a mutant may comprise at least one of a deletion, insertion, inversion or substitution, always provided that the resulting miR is capable of interacting with the 3' UTR to inhibit or prevent translation of the associated coding sequence. Enhanced homology with the 3' UTR is preferred. A variant will generally be a naturally occurring mutant, and will normally comprise one or more substitutions.

Particularly preferred stretches of the microRNA of the present invention correspond to the so-called "seed" sequences.

It will be appreciated that reference to any sequence encompasses mutants and variants thereof, caused by substitutions, insertions or deletions, having levels of sequence homology (preferably at least 60%, more preferably at least 70%, more preferably at least 80%, more preferably at least 90%, more preferably at least 95%, more preferably at least 99%, and most preferably at least 99.5% sequence homology), or corresponding sequences capable to hybridising to the reference sequence under highly stringent conditions (preferably 6x SSC).

The antisense RNAs of the present invention may be provided in any suitable form to the target site. In this respect, the target site may be *in vivo*, ex *vivo*, or *in vitro*, for example, and the only requirement of the antisense RNA is that it interacts with the target 3' UTR sufficiently to be able to inhibit or prevent translation of the CXCR4 ORF.

The antisense RNA may be provided directly, or a target cell may be transformed with a vector encoding the antisense RNA directly, or a precursor therefor. Suitable precursors will be those that are processed to provide a mature miR, although it is not necessary that such precursors be transcribed as long primary transcripts, for example.

Where the antisense RNA is provided directly, then this may be provided in a stabilised form such as is available from Dharmacon (www.dharmacon.com, Boulder, CO, USA).

A large number of microRNAs are known from WO 2005/013901, the patent specification of which alone is over 400 pages. However, no specific function is provided therefor.

Thus, although microRNAs are known, we are the first to establish that naturally-occurring RNA sequences, in particular miR-146a, or inhibitors thereof, are in fact capable of modulating the expression of CXCR4 protein.

Insofar as miR-146a is known, and any stabilised versions thereof, such as provided by Dharmacon are known, then the present invention does not extend to these compounds *per se*. However, the present invention extends to these and all other antisense RNAs provided by the present invention, for use in therapy and other processes.

More particularly, the present invention provides the use of antisense RNA specific for all or part of the 3' untranslated region of CXCR4 protein mRNA in therapy.

The nature of the therapy is any that is affected by expression of CXCR4 protein. In particular, antisense RNAs of the present invention may be used in the treatment of CXCR4-dependent tumours, as well as other CXCR4-dependent, such as HIV and RA.

Solid, non-diffuse tumours may be targeted by direct injection of the tumour with a transforming vector, such as lentivirus, or adenovirus. If desired, the virus or vector may be labelled, such as with FITC (fluorescein isothiocyanate), in order to be able to monitor success of transformation.

Thus, it is also preferred that the present invention is used in the modulation of Megakaryopoiesis.

For the treatment of a more diffuse condition, then systemic administration may be appropriate, and antisense RNA may be administered by injection in a suitable vehicle, for example.

Levels of antisense RNA to be administered will be readily determined by the skilled physician, but may vary from about 1 μg/kg up to several hundred micrograms per kilogram.

The present invention further provides miR-146a inhibitors, and their use in therapy. These are referred to as "sense inhibitors" in that they are complementary, at least in part, to the antisense miRNA of the present invention.

Also provided is the use of a sense or antisense polynucleotide according to present invention in the manufacture of a medicament for the treatment or prophylaxis of the conditions specified herein.

Suitable inhibitors for miR-146a include antibodies and sense RNA sequences capable of interacting with these miRs. Such sense RNAs may correspond directly to the concomitant portion of the 3' UTR of CXCR4 mRNA, but there is no requirement that they do so. Indeed, as miRs frequently do not correspond entirely to the 3' UTR that they target, while the existence of dsRNA often leads to destruction of the target RNA, then it is a preferred embodiment that the inhibitor of miR-146a is entirely homologous for the corresponding length of miR-146a. The length of the inhibitor need not be as long as miR-146a, provided

that it interacts sufficiently at least to prevent either of these miRs interacting with the 3' UTR or CXCR4 mRNA, when so bound.

Conditions treatable by miR-146a inhibitors include those associated with downmodulation of CXCR4, thereby requiring upregulation of CXCR4.

Preferred methods of delivery of the antisense miRNA or sense inhibitors may be by any gene therapy method known in the art, as will be readily apparent to the skilled person. Such methods include the so-called "gene-gun" method or delivery within viral capsids, particularly adenoviral or lentiviral capsids encapsulating or enclosing said polynucleotides, preferably under the control of a suitable promoter.

Preferred means of administration by injection include intravenous, intramuscular, for instance. However, it will also be appreciated that the polynucleotides of the present invention can be administered by other methods such as transdermally or per orally, provided that they are suitably formulated.

Also provided is a "test kit" capable of testing the level of expression of the CXCR4 protein such that the physician or patient can determine whether or not levels of the CXCR4 protein should be increased or decreased by the sense or antisense sequences of the present invention.

The present invention also encompasses a polynucleotide sequence, particularly a DNA sequence, which encodes the microRNAs of the present invention, operably linked to a suitable first promoter so that the MicroRNAs can be transcribed *in vivo*. Similarly, the present invention also provides a polynucleotide, particularly DNA, providing polynucleotides encoding the sense microRNA inhibitors of the present invention, also operably linked to a suitable second promoter for *in vivo* expression of said sense microRNA inhibitors.

In particular, it is also preferred that the first and second promoters mentioned above can be controlled by a third element, such that the level of expression of the antisense microRNA and the level of expression of the sense microRNA inhibitors can be controlled in a coordinated manner. In this regard, it is preferred that a feedback mechanism is also included for establishing this level of control.

Chimeric molecules are also provided, consisting of a polynucleotide according to the present invention, i.e. the antisense MicroRNAs or the sense microRNA inhibitors, linked to a second element. The second element may be a further polynucleotide sequence or may be a protein sequence, such as part or all of an antibody. Alternatively, the second element may have the function or a marker so that the location of microRNAs can be followed.

Thus, miR-146a and antagomirs thereof are useful in controlling or mediating expression of CXCR4 and controlling or mediating Megakaryopoiesis.

Here, we have shown that miR-146a masters Mk differentiation by targeting the CXCR4 receptor. This is surprising because miR-146a was only previously known in carcinogenesis (He et al., 2005) and in the regulation of the innate immune response (Taganov et al., 2006). However, our results now indicate that miR-146a plays a pivotal role in the control of CXCR4 expression in Mk differentiation. Although bioinformatic algorithms suggest that several miRs putatively target CXCR4 (see Results), their absence or specific expression profile in Mk cell lines suggests that they do not control CXCR4 protein levels in Megakaryopoiesis. In contrast, we demonstrated that miR-146a interacts directly with in the 3' UTR CXCR4 mRNA region and that CXCR4 is a specific target of miR-146a in the Mk lineage. Thus, our study represents the first report showing a role for a specific miR in the regulation of CXCR4 expression.

We identified a multistep regulatory mechanism in normal Megakaryopoiesis, including the transcription factor PLZF, miR-146a and CXCR4, i.e., the receptor for stromal cell-derived factor-1. In leukemic cell lines PLZF overexpression downmodulated miR-146a and upregulated CXCR4 protein, whereas PLZF knock-down induced opposite effects.

In vitro assays indicated that: (a) miR-146a targets the CXCR4 mRNA, impeding its translation; (b) PLZF interacts with the miR-146a promoter, inhibiting its transcriptional activity. In unilineage Megakaryopoietic (Mk) culture of cord blood $CD34^+$ progenitors, PLZF expression was upregulated, whereas miR-146a expression declined and CXCR4 protein level inversely increased. In functional experiments miR-146a transfection impaired proliferation and differentiation/ maturation of Mk cells, as well as Mk colony formation. Altogether, our data indicate that normal Megakaryopoiesis is controlled by a novel cascade

pathway, whereby an increased PLZF level suppresses transcription of miR-146a, hence activating CXCR4 translation.

The CXCR4 receptor is involved in the process of cancer metastasis (Wang et al. 2006), mediates HIV entry into CD4+ lymphocytes (Littman DR, 1998) and plays a major role in the pathogenesis of rheumatoid arthritis (Nanki et al. 2000). Particularly, the interaction between SDF-1a and CXCR4 has a pivotal role in the directional migration of hematopoietic and epithelial cancer cells during the metastatic process (Bürger and Kipps, 2006): therefore, small CXCR4 antagonist molecules have been developed for potential use in metastasis therapy (Tamamura and Fujii, 2005). In view of the capacity of anti-miR antisense oligonucleotides ("antagomirs") to exert effective in vivo actions (Krutzfeldt et al 2005; Carè et al., 2007), our results indicate that these anti-miR-146a molecules may represent a key tool for molecular therapy in the above clinical settings.

The invention will now be described in relation to the Figures and accompanying Examples. The Figures show:

Figure 1. PLZF expression induces miR-146a downmodulation and CXCR4 protein upmodulation in K562-PLZF cells

(**a**) Western blot analysis of PLZF protein expression in K562 cells infected with control (LXSN) or PLZF vectors. Actin is the internal control. One representative experiment (left panel) and PLZF protein expression ± SEM values from 3 independent clones of K562-PLZF cells (right panel) are shown. (**b**) Northern blot analysis of miR-146a expression in K562-LXSN and K562-PLZF cells. tRNA is shown for normalization. One representative experiment (left panel) and the ratio of miR-146a/ tRNA expression ± SEM values from 3 independent clones of K562-PLZF cells (right panel) are shown. ***P< 0.001 when compared to LXSN. (**c**) Real time PCR analysis of CXCR4 mRNA expression in K562-LXSN and K562-PLZF cells. The error bars represent the mean ± s.d. (n = 3). (**d**) Flow cytometry analysis of CXCR4 protein expression, performed on permeabilized K562-LXSN and -PLZF cells stained with PE-conjugated anti-CXCR4 mAb (CXCR4 PE, clear bar) or isotype control (shaded). The Geometric Mean Fluorescence Intensity Ratio (MFIR) of one representative experiment (middle panel) and mean CXCR4 expression ± SEM values from 3 independent clones (right panel) are shown. **P< 0.01 when compared to LXSN.

(**d**) *Left panel*: Growth curve of K562-LXSN and K562-PLZF cultures treated with the Mk inducer, PDB, compared to untreated cells (C). Mean ± SEM values from 3 independent

experiments is shown, *P< 0.05 when compared to LXSN Cont.+/- PDB; *Right panel*: FACS analysis of Mk markers CD61, CD41 and CD9 antigens expression at sequential days in cultures from cells treated with PDB or untreated (C).

Regulation loop among PLZF, miR-146a and CXCR4 protein expression in leukemic cells: **(e)** *Left panel*: Real time PCR analysis of PLZF mRNA expression level; *right panels*: Western blot analysis of PLZF protein level, in PLZF-siRNAs transfected HEL cells (siR) as compared to control-siRNAs transfected HEL cells (C). **(f)** Northern blot analysis of miR-146a expression in PLZF-siRNA transfected HEL cells (siR) as compared to control siRNA-transfected HEL cells (C). tRNA was used for normalization. **(g)** *Left panel*: Real time PCR analysis of CXCR4 mRNA expression in C and siR HEL samples. The error bars represent the mean ± s.d. (n = 3); *right panels*: FACS analysis of CXCR4 protein level in siR-compared to C-HEL samples. A representative experiment out of three (left panel) and mean ± SEM of 3 independent experiments (right panel) are shown.

Figure 2. Regulation loop among PLZF, miR-146a and CXCR4 protein expression in leukemic cells

(a) Real time PCR analysis of PLZF mRNA expression level in PLZF siRNAs-transfected HEL cells (siR) as compared to control siRNAs-transfected HEL cells (C).

(b) Northern blot analysis of miR-146a expression in PLZF siRNA-transfected HEL cells (siR) as compared to control siRNA-transfected HEL cells (C). tRNA was used for normalization.

(c) Real time PCR analysis of CXCR4 mRNA expression in C and siR HEL samples.

(d) FACS analysis of CXCR4 protein level in siR-compared to C-HEL samples. A representative experiment out of four is shown.

Figure 3. PLZF, miR-146a and CXCR4 expression in purified HPCs induced to megakaryocytic (Mk) differentiation

(a) Immunofluorescence analysis of PLZF protein expression (α-PLZF) at the indicated sequential days in HPCs grown in liquid-phase unilineage Mk cultures. (-): no primary antibody negative control. The upper panels show the phase contrast microscopy fields.

(b) RT-PCR analysis of pre-miR-146a expression; and

(c) real time PCR analysis of miR-146a expression, performed at the indicated sequential days, in HPCs grown in liquid-phase unilineage Mk cultures. GAPDH is presented for normalization.

(**d**) Real time PCR analysis of CXCR4 mRNA expression performed in HPCs during Mk differentiation. **A, B, C** and **D** show one representative experiment out of three.

(**e**) Flow cytometry analysis of CXCR4 protein expression in fixed and permeabilized HPCs during liquid-phase unilineage Mk cultures, stained with a PE-conjugated anti-CXCR4 mAb (shaded) or isotype control (clear). The Geometric Mean Fluorescence Intensity Ratio (MFIR) of one representative experiment is indicated.

Figure 4. miR-146a directly interacts with CXCR4 mRNA-3'UTR

(**a**) Flow cytometry analysis of CXCR4 protein expression in miR-146a transfected Jurkat cells. The transfected increasing amounts of miR-146a are indicated. CD3 expression was evaluated as a negative control.

(**b**) Real time PCR of CXCR4 mRNA in the same cells. One representative experiment out of three is shown.

(**c**) Sequence of the three putative miR-146a binding sites (*in red*) in the 3' UTR-CXCR4 mRNA region.

(**d**) Luciferase targeting assay on lysates from Phoenix cells co-transfected with miR-146a or non targeting RNA and psiCHECK 2 empty vector (E), or with the same vector containing the full length 3' UTR of CXCR4 mRNA (FL) or fragments of the CXCR4 3'UTR containing Site 2 (S2), Site 3 (S3), Sites 2 and 3 (S2/3), a mutated S2 site (Mut2) or a mutated S3 site (Mut3). Values represent the percentage of the luciferase activity obtained when co-transfecting the nontargeting oligonucleotide. Mean ± SEM values from three separate experiments. ** $P < 0.01$ where compared to control group.

Figure 5. PLZF binds and represses miR-146a promoter

(**a**) Scheme of the miR-146a promoter on chromosome 5 showing the PLZF binding site in the region upstream the pre-miR-146a. The arrows indicate the position of the primers used for the ChIP experiments.

(**b**) Chromatin immunoprecipitation from lysates of HEL cells with an anti-PLZF mAb (α-PLZF), an unrelated antibody (α-c-abl) or no antibody (no-Ab). Immunoprecipitated chromatin was analysed by PCR amplification of the miR-146a promoter region surrounding the *PLZF* binding site (Prom 146a) or a region that does not contain *PLZF* binding sites (Control Prom) or a GAPDH coding region, to detect non relevant DNA sequences. A sample representing 0.02% of total chromatin (input) was included in the PCR analysis.

(**c**) Promoter activity assay of the pre-miR-146a upstream DNA sequence (Prom 146a) cloned in a promoterless luciferase reporter vector (pGL3Basic) and transfected into Phoenix cells in the presence (clear bars) or absence (black) of a PLZF expression vector. Vector indicates the empty reporter vector.

(**d**) Promoter activity assay of the pre-miR-146a upstream DNA sequence (Prom 146a), as in C, or the same region with a mutated PLZF binding site (shown on the right) (Mut. Prom 146a), cloned in a reporter vector containing a minimal promoter (pGL3Prom) and transfected with or without a PLZF expression vector.

The data in **c** and **d** are reported as percentage of the value obtained with the promoter constructs in the absence of the PLZF vector. Mean values of three independents experiments.

Figure 6. Ectopic miR-146a expression in Mk cells downmodulates CXCR4 protein expression and impairs cell growth and Mk differentiation-maturation

(**a**) Growth curve of Mk culture transfected with miR-146a (miR-146a), or a control oligonucleotide (Cont. oligo), compared to untreated Mk cells (Control Mk). Mean ± SEM values from 3 independent experiments. **P< 0.01 when compared with control oligonucleotide transfected cells.

(**b**) *Left panel*: real time PCR analysis of CXCR4 mRNA expression in unilineage Mk culture transfected on day 1 (arrow) with miR-146a oligonucleotide (miR-146a), or non targeting oligonucleotide (Cont. oligo), compared to control Mk culture (Control Mk). *Right panel:* CXCR4 protein expression, evaluated at sequential days by flow cytometry in Mk culture in the miR-146a, Cont. oligo and Control Mk groups. The cells were permeabilized and stained with a PE-conjugated anti-CXCR4 mAb (shaded bar) or isotype control (clear). The Geometric Mean Fluorescence Intensity Ratio (MFIR) of one representative experiment is indicated.

(**c**) Morphological analysis of the differentiation and maturation of Mk cells transfected with miR-146a (miR-146a), or control oligonucleotide (Cont. oligo) or untreated (Control Mk), collected at day 14 and stained with May-Grünwald-Giemsa for evaluation of cells featuring polylobated nuclei (*Left panel*). Mean ± SEM values from 3 independent experiments. **P< 0.01 when compared with Cont. oligo transfected cells.

(**d**) FACS analysis of CD61 and CD41 antigens expression at sequential days in Mk cultures from cells transfected with miR-146a (miR-146a), or control oligonucleotide (Cont. oligo) or untreated (Control Mk).

(e) Megakaryocytic (upper panel), erythroid and granulo-monocytic (left and right lower panel respectively) colony-forming capacity of HPCs transfected with miR-146a (miR-146a), or control oligonucleotide (Cont. oligo) or untreated HPCs cells (C). Mean ± SEM values from 3 independent experiments. **P< 0.01 when compared with Cont. oligo transfected cells.

Figure 7. Inhibition of PLZF expression by siRNA treatment impairs megakaryopoiesis

(a) Growth curve of Mk culture cells transfected with siRNA targeting PLZF mRNA (PLZF-siRNA) or non targeting control siRNA (C-siRNA), as compared to untreated Mk cells (Control Mk). Mean ± SEM values from 3 independent experiments.

(b) Real time PCR analysis of PLZF mRNA expression (*Left panel*) (mean ± SEM values from 3 independent experiments) and RT-PCR analysis of pre-miR-146a expression (*Right panel*) (results from a representative experiment out of three are presented) in unilineage Mk culture transfected on day 1 (arrow) with PLZF-siRNA, as compared to control C-siRNA Mk culture.

(c) Flow cytometry analysis of CXCR4 protein expression, at sequential days in Mk culture in the PLZF-siRNA, C-siRNA and Control Mk groups. The Geometric Mean Fluorescence Intensity Ratio (MFIR) indicates mean ± SEM values from 3 independent experiments. **P< 0.01 when compared with C-siRNA transfected cells.

(d) Morphological analysis of the differentiation and maturation of Mk cells transfected with PLZF-siRNA (PLZF), or control C-siRNA (C) or untreated (Cont. Mk), collected at day 14 and stained with May-Grünwald-Giemsa for evaluation of cells featuring polylobated nuclei (*Left panel*). Mean ± SEM values from 3 independent experiments. **P< 0.01 when compared with control C-siRNA transfected cells.

(e) FACS analysis of CD61 and CD9 antigens expression at sequential days in Mk cultures from cells transfected with PLZF-siRNA, or control C-siRNA or untreated (Control MK).

(f) Megakaryocytic (upper panel), erythroid and granulo-monocytic (left and right lower panel respectively) colonies generated by HPCs transfected with PLZF-siRNA (PLZF) or control C-siRNA (C), or by untreated HPCs (Cont.) (C). Mean ± SEM values from 3 independent experiments. **P< 0.01 when compared with control C-siRNA transfected cells.

Figure 8: MicroRNA expression profiling in leukemic cell lines

Northern Blot analysis of human microRNAs expression in leukemic cell lines: K562-PLZF compared to K562-LXSN cells, HEL and Jurkat (JURK). MiRs expressed in the cell lines are shown (except for miR-9). MiR-1b, 7b, 9, 122a, 124, 125a, 129, 133, 139*, 148a, 153, 183, 184, 191, 200b, 205, 217, 221, 222, 224*, 281-1, 281-2, 381*, 410* were not expressed in these cell lines. tRNA was used for RNA normalization. Representative results from three independent experiments are shown,

* Putative miRs targeting CXCR4 mRNA (www.TargetScan.org).

Figs 9-11 are described in more detail in the Examples below.

EXAMPLES

EXPERIMENTAL PROCEDURES

Cell lines. K562, HEL, Jurkat and Phoenix cells were cultured using standard methods. Transduced K562-LXSN and three selected clones 1, 2, 3 of K562-PLZF cells were prepared as described[32].

PDB treatment of K562 cells. Cells were incubated for 4 days in the presence of PDB (Phorbol Dibutyrate, Sigma St Louis, USA) as described in[35] and analyzed for Mk membrane markers (CD9, CD41, CD61) expression by Flow cytometry analysis, as described below.

Unilineage and clonogenic Mk culture. Collection of CB, isolation of $CD34^+$ cells, unilineage Mk culture and morphology analysis were performed as described[35]. For clonogenicity assays, after miR-146a or nontargeting oligonucleotides transfection, $1x10^3$ HPCs were plated in methylcellulose medium containing a saturating concentration of TPO[35]. HPC colonies were scored on days 10-14. Multilineage cultures of $CD34^+$ HPCs were performed as previously described[37].

Western blotting

Western blot analysis to evaluate PLZF protein expression was performed using the anti-PLZF mAb (Oncogene Research Products, Boston, MA)[32].

MiR expression analysis

Northern blotting of 25 μg of total RNA and miRs expression analysis were performed as described[11].

Quantitative real-time (qRT)-PCR analysis of miR-146a was performed by TaqMan technology, using the kit ABI PRISM 7700 DNA Sequence Detection System specific for miR-146a reverse transcription and PCR analysis (Applied Biosystems, Foster City, CA) according to the manufacturer's procedure.

Reverse transcription (RT)-PCR analysis for pre-miR-146a was performed using random primers-RT kit (Invitrogen), according to the manufacturer's procedure. PCR analysis was performed with the following pre-miR-146a primers: forward - SEQ ID NO. 9; Reverse - SEQ ID NO. 10. PCR products were analyzed by Southern blot using a miR-146a antisense ^{32}P-labelled probe: SEQ ID NO. 11. Normalization was performed using GAPDH RT-PCR analysis[32].

qRT-PCR analysis for GAPDH, PLZF (ZNF145), CXCR4 was performed according to standard procedures[32]. Commercial ready-to-use primers/probe mixes were used (Assays on Demand Products, Applied Biosystems, Foster City, CA).

Flow cytometry analysis

To evaluate total CXCR4 protein expression, cells were fixed and permeabilized (Permi-Fix, BD/Pharmingen) before incubation with either PE-conjugated anti-CXCR4 mAb (clone 1245, BD/Pharmingen) or a control PE-conjugated mAb of the same isotype. The cells were analyzed using the FACSCAN flow cytometer and the Cell Quest software (BD) for acquisition and analysis.

The reported mean fluorescence intensity (MFIR) indicates the ratio between the geometric mean fluorescence values observed in CXCR4 labeled cells and cells labeled with a negative control, gated on FSC/ SSC dot plot to select viable cells. The same procedure was followed for CD3 detection, using a PE-conjugated anti-CD3 mAb (clone UCHT1, BD/Pharmingen).

Flow cytometric analysis of K562-LXSN and K562-PLZF cells untreated or treated with PDB, and Mk cultures, were performed as described[35].

Morphological and immunofluorescence analysis

For morphological analysis, HPCs were harvested on different days of Mk unilineage culture, transferred to glass slides by cytospin centrifugation and stained with May-Grünwald-Giemsa.

For immunofluorescence labelling, cells were fixed on glass slides in absolute methanol at room temperature for 5 min, permeabilized in absolute acetone at -20° C for 2 minutes and washed in PBS. The slides were then incubated 30 min. with the anti-PLZF mAb (1:100 in PBS/BSA, final dilution 10 μg/ml), washed in PBS and incubated for 30 min with FITC-labeled $F(ab')_2$ fragments of affinity goat purified α-Mouse IgG 1:40 (Dakopatts, Copenhagen, Denmark). After extensive wash in PBS, slides were mounted with antifade glycerol mounting medium (Moulecular Probes, Eugene, OR, USA) and analyzed under an inverted Olympus FV500 equipped for confocal microscopy.

ChIP assay

$3x10^6$ HEL cells were crosslinked by addition to the culture medium of formaldehyde at 1% final concentration and incubation for 10 min at 37°C. After sonication, ChIP assay kit (Upstate USA, Charlottesville, VA) was used according to the manufacturer's procedure and protein-DNA complexes were immunoprecipitated overnight with the anti-PLZF mAb, an anti c-abl mAb (Oncogene Research Products, Boston, MA) or protein-A sepharose only. A genomic fragment of 128 bp containing the PLZF binding site in the pre-miR-146a

upstream region (Prom 146a) (*Ensembl* Genomic Gene ID ENSG00000199083) was amplified by PCR using primers flanking the PLZF site: forward SEQ ID NO. 12; reverse SEQ ID NO. 13. PCR products were Southern blotted and hybridized with the internal probe ^{32}P-labeled oligonucleotide SEQ ID NO. 14. As control, a flanking 140 bp genomic region, not containing any PLZF site, was amplified by PCR and analyzed using the following primers: sense SEQ ID NO. 15; antisense SEQ ID NO. 16 and internal probe SEQ ID NO. 17. Non relevant cellular DNA sequences were detected by amplification of a GAPDH coding region using primers and PCR conditions as described[32].

Promoter assay

A 475 bp DNA fragment of Prom 146a was PCR-amplified from genomic DNA using the primers forward SEQ ID NO. 18 and reverse SEQ ID NO. 19, and cloned upstream to the luciferase gene into the pGL3Basic (pGL3Basic/Prom 146a) and pGL3Promoter (pGL3Prom/Prom 146a) vectors (Promega). By mutagenesis of the *PLZF* site into the pGL3Prom/Prom 146a vector using, according manufacturer's instructions, the QuickChange Site-Directed mutagenesis kit (Stratagene), we prepared the PLZF mutated Prom 146a vector (pGL3Prom/Mut. Prom 146a). The pcDNA3/PLZF (PLZF) was previously reported[31]. All vectors were checked by automated sequencing.

In luciferase assay experiments, Phoenix cells were transfected using Lipofectamine 2000 (Invitrogen), with a *Renilla* luciferase vector (50 ng), together with luciferase vectors described above (0,8 ug) and, where indicated, with the pcDNA3/PLZF. Luciferase activity was measured 48 hr post-transfection with the Dual Luciferase Reporter System (Promega, Madison, WI, USA) according to the manufacturer's instructions, by using Microlite TLX1 (Dynatech Laboratoires, Chantilly, CA) and then normalized for *Renilla* Luciferase activity. Data are presented as mean values, obtained from at least three independents experiments.

Silencing of PLZF or CXCR4 expression by siRNAs

Two double-stranded small interfering PLZF-siRNA sequences (NM_001018011), or CXCR4-siRNAs sequences (NM_003467) and the control C-siRNA sequence with no homology to the human genome were purchased from Dharmacon, Lafayette, CO. Cells were co-transfected with 90 nM PLZF-siRNA or CXCR4-siRNAs or C-siRNA and FITC-conjugated nontargeting oligonucleotide using Lipofectamine according to the manufacturer's instruction. After 48 hrs, cells were harvested and analysed for PLZF, miR-146a and CXCR4 mRNA and protein expression as described above.

Transfection of miR-146a oligonucleotide

Stability-enhanced miR-146a oligonucleotide (miR-146a), control nontargeting RNA oligonucleotide (Cont. oligo), antagomir-146a (α-miR-146a), unrelated antagomir-133a (Cont. α-miR) and FITC-conjugated nontargeting oligonucleotide were purchased from Dharmacon. Cell lines were transfected using Lipofectamine and collected for analysis 48 hrs after transfection. HPCs were transfected at day 0 or 1 of Mk cultures and collected at sequential times through Mk differentiation/maturation.

Luciferase target assay

Plasmids and constructs We used the psiCHECK 2- 3'UTR vector (Promega, Madison, WI USA) to clone, downstream to the *Renilla* luciferase gene, the full length 3' UTR of CXCR4 mRNA (FL wild type, wt vector) (NM_001008540, from position 1450 to 1862), which includes 3 putative miR-146a binding sites (Site 1: from 1452 to 1473 bp; Site 2: from 1575 to 1596 bp; Site 3: from 1800 to 1821 bp). In the same plasmid we cloned CXCR4 mRNA fragments: S1 (1450 to 1573 bp, including Site 1), S2 (1554 to 1775 bp, including Site 2), S3 (1756 to 1862 bp, including Site 3), S2/3 (1554 to 1862 bp, including both Sites 2 and Site 3) and Cont. (1621 to 1750 bp) a fragment without putative miR-146a binding site to use as a control.

By mutagenesis (QuickChange Site-Directed mutagenesis kit ,Stratagene) of the Site 1, Site 2 and/or Site 3 in the reporter vectors FL wt and S2, S3, we prepared the constructs FL-Mut1, FL-Mut2, FL-Mut3, FL-Mut2/3 and MutS2, MutS3 respectively. We used the following primers: for the mutation of Site 1, SEQ ID NO. 20 5'-GACCAATATTGTA*GCGGCCGC*A TTGCTTGTTGGATTT-3' (from 1452 to 1489); Site 2, SEQ ID NO. 21 5'-GCAGGACCTGTGGCCA*GCGGCCGC*GTTGCTGTATGTCTCG-3' (from 1573 to 1612); Site 3, SEQ ID NO. 22 5'-ATAGAAATGCTGGTTT*GCGGCCGCTCA*GGAGTGGG TTGATTTC-3' (from 1795 to 1837).

Luciferase assay. Phoenix cells were cotransfected with 75 ng of psiCHECK-2 reporter vectors and 60 pmol of either the nontargeting RNA oligonucleotide or stability-enhanced miR-146a oligonucleotide, using Lipofectamine. After 48 hrs, cells were lysed with Passive Lysis Buffer (Promega, Madison, WI USA), and their *Renilla* luciferase activity was measured by using the Femto-master FB 12 (Zylux, Oak Ridge, TN). The relative reporter activity was obtained by normalization to the psiCHECK-2- 3' UTR/nontargeting oligonucleotide, cotransfection.

Transfection of miR-146a and CXCR4-3'UTR mutated expression vectors

Plasmids constructs. We used the pcDNA 3.1 expression vector (Promega, Madison, WI USA) to subclone into Hind III /Eco RI sites, the full length CXCR4 coding sequence and its 3'UTR region (CXCR4-3'UTR wild type, CXCR4-wt vector). The oligonucleotides used for PCR amplification were: CXCR4-forward SEQ ID NO. 23 and 3'UTR-reverse SEQ ID NO. 24. By mutagenesis (QuickChange Site-Directed mutagenesis kit ,Stratagene) of the 3' UTR region of CXCR4-wt vector into both Sites 2 and 3, we prepared the construct CXCR4-3'UTR/S2/S3 mutated (CXCR4-Mut2/3) . We also subcloned pre-miR-146a in pcDNA 3.1 vector (miR-146a). All vectors were checked by automated sequencing. The transfection of CXCR4-wt, CXCR4-Mut2/3 or miR-146a were performed in K562-LXSN

and overexpression tested by FACS analysis and Northern blot analysis respectively (data not shown).

Transfection assays. K562 cells were cotransfected with 100 ng of pcDNA 3.1 empty vector, CXCR4-wt or CXCR4-Mut.2/3 vector and miR-146a vector, using Lipofectamine. After 48 hrs, cells were treated with PDB and analyzed 2/4 days later for CXCR4 and Mk markers expression, as compared to untreated cells.

RESULTS

PLZF, miR-146a and CXCR4 protein expression correlate in hematopoietic cell lines

In previous studies we transduced K562 cells with PLZF (K562-PLZF cells) and characterized them, as compared to control cells transduced with empty vector (K562-LXSN)[32] (Fig.1a). In three clones of K562-PLZF (clones 1, 2, 3)[32] the miRNA expression profile was evaluated by Northern blot (Fig 8). Among several miRNAs of interest, we found that miR-146a expression is consistently downmodulated in K562-PLZF cells (Fig. 1b, PLZF), compared to control ones (Fig. 1b, LXSN and Fig. 8), suggesting that miR-146a may be a target of PLZF. In K562-PLZF cells, CXCR4 mRNA expression is not significantly modulated, whereas CXCR4 protein level is upregulated, as indicated respectively by quantitative Real Time (qRT-) PCR (Fig. 1c, left panel) and FACS evaluation (Fig. 1c, middle and right panels). Analysis of the sequence of the CXCR4 gene promoter did not reveal any functional PLZF binding site, suggesting that CXCR4 is not a direct target gene of PLZF. On the other hand, the discrepancy between CXCR4 mRNA and protein levels in K562-PLZF cells suggested that CXCR4 mRNA may be targeted by miR-

146a: this hypothesis was supported by a single bioinformatic algorithm (http://microrna.sanger.ac.uk/).

Importantly, the algorithm www.TargetScan.org suggested that CXCR4 mRNA is a putative target of other miRNAs, including miR-*9, -93, -224, -381, -410*. However, miR-*9, -224, -381, -410* were not expressed in hematopoietic cell lines; in the case of miR-*93*, we found no correlation with the levels of CXCR4 protein expression (Fig. 8 and legend). miR-23b is not consistently regulated in different K562-PLZF clones (Fig.8), does not target CXCR4 3'UTR and does not modify the expression of CXCR4 protein when overexpressed in Jurkat cells (Fig 9).

To further investigate the regulatory network involving PLZF and miR-146a in the control of CXCR4 expression, we used the PLZF positive cell line HEL to downmodulate PLZF expression by small interfering RNAs (siRNA), as shown by qRT-PCR (Fig 10a left panel) and Western blot analysis (Fig 10a middle and right panels), and examined the effects on miR-146a, and CXCR4 expression at mRNA and protein level. PLZF siRNA (siR), but not control siRNA (C), promoted miR-146a expression (Fig. 10b), while inducing CXCR4 protein downmodulation (Fig. 10c, middle and right panels), without any significant change of CXCR4 mRNA level (Fig.10, left panel).

Overall, our results showed an inverse correlation between PLZF and miR-146a level, as well as between miR-146a and CXCR4 protein expression in both K562-PLZF cells and PLZF siRNA-treated HEL cells.

K562 cells induced to Mk differentiation: a model system to investigate the role of the PLZF/miR-146a/CXCR4 receptor pathway in megakaryopoiesis.

We then analyzed the growth and Mk differentiation of K562-PLZF cells, as compared to K562-LXSN control[33]. Their proliferation rate was slightly lower than in control cells (Fig 1e, left panel). In three clones of K562-PLZF cells the expression of the Mk differentiation antigens CD61, CD41 and CD9 was increased, as compared to the

control cells (right panel). Upon Mk differentiation induced by phorbol dibutyrate (PDB)[34], PLZF expression was enhanced (Fig. 1d, left panel), while the level of pre-miR-146a was reduced (middle panel) and that of CXCR4 protein was increased (right panel). PDB treatment impaired cell proliferation (Fig 1e, left panel), while inducing the expression of CD41, CD61, CD9 antigens at significantly higher levels in K562-PLZF than in control cells (right panel). Altogether, these data indicate that PLZF potentiates the Mk differentiation of K562 cells and is coupled with diminished miR-146a and enhanced CXCR4 protein expression. We hence postulate that these cells represent a useful model system for evaluation of the role of the PLZF/miR-146a/CXCR4 complex in Mk differentiation.

PLZF, miR-146a and CXCR4 expression in unilineage Mk culture

Our observations in K562 cells prompted us to evaluate the expression of PLZF, miR-146a, CXCR4 mRNA and protein, at sequential days, in human cord blood (CB) $CD34^+$ HPCs induced to unilineage differentiation and maturation through the Mk pathway in serum-free liquid suspension culture[35].

In agreement with the effects of PDB in K562 cells, in these Mk cultures, PLZF protein expression is gradually upregulated starting from day 1-3, as shown by anti-PLZF immunofluorescence and Western blot analysis (Fig. 2a). In parallel, we analyzed pre-miR-*146a* (pre-miR-146a) and miR-146a expression by RT-PCR (Fig. 2b, left panel) and qRT-PCR (Fig. 2b, right panel) analysis respectively. The data showed a high level of pre-miR-146a and miR-146a expression in quiescent $CD34^+$ cells (day 0), followed by a gradual, marked downmodulation in the day 1-3 period, which persisted thereafter. Thus, we observed an inverse correlation between PLZF protein and miR-146a expression levels during Mk differentiation/maturation (Fig. 2d).

In the same Mk cultures, the CXCR4 mRNA level, analysed by RT-PCR, showed a relatively mild fluctuation: specifically, it was elevated at day 0 and 1, lower at day 3-8 and

then elevated again at day 10 (Fig. 2c, left panel). These fluctuations are seemingly mediated by transcriptional regulatory mechanisms. Conversely, FACS analysis showed that total CXCR4 protein expression was markedly upregulated, peaking at day 3 and sustainedly enhanced through day 10 (Fig. 2c, right panel). The rise of CXCR4 protein, in presence of a decreased mRNA, suggests that the sharp decline of miR-146a effectively unblocks CXCR4 mRNA translation.

Altogether, these data indicate that during Mk differentiation, (i) miR-146a level was inversely related to PLZF expression (Fig. 2d); (ii) miR-146a expression was inversely related to CXCR4 protein level (Fig. 2e). The results hence suggest a transcriptional control by PLZF on miR-146a expression, coupled with a posttranscriptional control of miR-146a on CXCR4 protein level.

CXCR4 is a direct target of miR-146a

To verify whether CXCR4 is a direct target of miR-146a, we over-expressed miR-146a in the Jurkat cell line, since it is negative for miR-146a expression (Fig. 8). We cotransfected the cells with: (a) a nontargeting scrambled oligonucleotide or miR-146a and (b) a FITC-conjugated non-targeting oligonucleotide used as a tracer for FACS analysis. 80% of the cells were FITC-positive (data not shown). FACS evaluation of CXCR4 protein expression revealed that CXCR4 protein was downmodulated by miR-146a in a dose-dependent way (Fig. 3a, right panel), without any significant modulation of CXCR4 mRNA expression (Fig. 3b). Conversely, CXCR4 level was unchanged in cells transfected with the nontargeting oligonucleotide (Fig. 3a, left panel). The inhibition of CXCR4 protein expression was time-dependent, with the lowest level at 48 h post-transfection (not shown). The specificity of these effects was confirmed by FACS analysis of the CD3 molecule, which was not modulated by the nontargeting oligonucleotide or miR-146a transfection (Fig. 3a).

To demonstrate the direct interaction between miR-146a and CXCR4 mRNA, we inserted downstream of a *Renilla* luciferase reporter cDNA the full length 3' UTR region of CXCR4 mRNA (FL wt), including three sequences complementary to miR-146a (Fig. 3c, Site 1, 2, 3), as well as the FL region containing a mutagenized S1, S2, S3 or S2/S3 binding sites (vectors FL-Mut1, FL-Mut2, FL-Mut3, FL-Mut2/3, respectively). We also inserted in the reporter vector, shorter fragments containing the single putative binding site 1, 2, 3, or 2 and 3 (vectors S1, S2, S3, S2/3, respectively) or their mutants (MutS2, MutS3) (Fig. 3d). Each vector was transfected in the miR-146a negative Phoenix cell line, together with miR-146a or a control non-targeting oligonucleotide (Cont. oligo). The luciferase activity of the FL wt, FL-Mut1, FL-Mut2, FL-Mut3, and also of the S2, S3 or S2/3 reporter vectors was markedly diminished after miR-146a cotransfection (Fig. 3d, miR-146a), while not affected by the non-targeting oligonucleotide (Fig. 3d, Cont. oligo). We did not notice any modulation of luciferase activity with the S1 reporter vector (Fig. 3d, Fragments/miR-146a) excluding the Site 1 as a miR-146a binding site. Importantly, we did not detect any modulation of luciferase activity after miR-146a cotransfection with the FL-Mut2/3, MutS2 or MutS3 reporter vector, containing a mutagenized S2/S3, S2 or S3 binding site respectively (Fig. 3d). The results hence indicated that miR-146a interferes with CXCR4 mRNA translation via direct interaction with the 3' UTR-FL (FL-wt), specifically via miR-146a binding of S2 and S3 sites.

PLZF is a transcriptional repressor of miR-146a expression

To investigate the potential interaction between PLZF and miR-146a expression, we analyzed the genomic region upstream the pre-miR-146a on chromosome 5 (ENSG00000199083). At position (-137; -117) we found the sequence SEQ ID NO. 25 5'-TAGACCTG***GTAC***TAGGAAGCA-3' (Fig. 3e), related to the previously described *PLZF* DNA-binding consensus[36].

We evaluated whether PLZF was recruited at the pre-miR-146a DNA upstream region (Fig. 3e) by chromatin immunoprecipitation (ChIP) experiments performed on the PLZF-expressing HEL cell line. Protein-DNA complexes were immunoprecipitated with either the anti-PLZF mAb, or no antibody, or the unrelated anti-c-abl mAb used as negative control (Fig. 3f, α-PLZF, no-Ab, α-c-abl respectively). The immunoprecipitates were then amplified by PCR using: (i) primers flanking the putative PLZF DNA-binding site in the Prom 146a region (Fig. 3f, Prom 146a); (ii) primers recognizing a region upstream to Prom 146a sequence, which does not contain any identifiable *PLZF* site (Fig. 3f, Control Prom). Our ChIP assays indicated that in HEL cells PLZF is associated to the region surrounding the putative *PLZF* binding site into the Prom 146a genomic region.

We then cloned this region in a luciferase reporter construct (pGL3Basic) and investigated its ability to promote transcription. The pGL3Basic/Prom146a was able to enhance by 30-fold the luciferase activity over pGL3Basic level, whereas PLZF expression was able to repress 40% of this effect (Fig. 3g, left panel). To further demonstrate that PLZF acts as a repressor on the newly identified PLZF-binding site described above, we cloned the region containing this site (Prom 146a) or a mutagenized version (Mut. Prom 146a) into a reporter vector containing an artificial minimal promoter (pGL3Promoter) and performed luciferase assays. PLZF was able to repress the luciferase activity detected for the Prom 146a vector, but not the activity of the Mut. Prom 146a vector (Fig. 3g, right panel).

Together our data identified a new *PLZF* DNA-binding site in Prom 146a and indicated PLZF as a direct transcriptional repressor of miR-146a.

Enforced expression of miR-146a in unilineage Mk cultures blocks CXCR4 protein expression and impairs cell proliferation and differentiation/maturation

We further evaluated whether ectopic expression of miR-146a affects the proliferation, differentiation and maturation of Mk cultures.

$CD34^+$ cells, seeded in unilineage Mk liquid suspension cultures, were co-transfected on day 1 with: (a) miR-146a or a nontargeting scrambled oligonucleotide and (b) a FITC-conjugated non-targeting oligonucleotide used as a tracer for FACS analysis. Transfection efficiency was 70% (data not shown). We sorted the FITC-positive cells by FACS and seeded them in unilineage Mk liquid suspension culture or in semisolid Mk medium for clonogenic analysis[35].

In Mk liquid culture, we analyzed at sequential times miR-146a transfected cells (miR-146a), as compared to nontargeting oligonucleotide transfected (Cont. oligo) and untransfected cells (Control Mk) (Fig. 4a). Analysis of CXCR4 mRNA expression by qRT-PCR (Fig. 4b, left panel) and CXCR4 protein level by FACS evaluation (Fig. 4b, right panel) indicated that miR-146a enforced expression induced protein downmodulation throughout megakaryopoiesis, without significantly affecting mRNA expression, indicating that CXCR4 is a target of miR-146a in the Mk lineage.

Notably, miR-146a-transfection induced a marked decrease in proliferation rate (Fig. 4a) and a marked reduction of cells with polylobated nuclei, suggesting a reduced Mk maturation (Fig. 4c). This was confirmed by analysis of CD61 and CD41 (Fig. 4d) and CD9 (not shown) antigens, whose expression was clearly lower in miR-146a-transfected cells than in control ones.

By clonogenic Mk[35] and multilineage[37] progenitor assay, we observed that miR-146a overexpression reduced the number of Mk colonies to ~ 30% of control value (Fig. 4e, upper panel), whereas it did not affect the number of BFU-E and CFU-GM colonies (lower panels), thus indicating that the miR-146a effect is Mk-specific.

Mk cultures were also transfected with siRNAs that specifically blocked PLZF expression (PLZF-siRNA) or nontargeting control siRNA (C-siRNA), together with FITC-conjugated double-stranded RNA. FITC positive cells were then sorted and cultured. Silencing of PLZF mRNA (Fig.5b, right panel) i) enhanced pre-miR-146a expression (Fig.

5b, left panel), ii) decreased CXCR4 protein level as evaluated by FACS (Fig. 5c), iii) decreased cell proliferation (Fig. 5a), and iv) impaired Mk differentiation/maturation, as shown by morphology (Fig. 5d), reduced expression of the surface antigens CD61, CD9 (Fig. 5e), CD41 and CD42b (not shown), as well as by clonogenic Mk and multilineage progenitor assay (Fig. 5f). These results are equivalent to those obtained when miR-146a, was transfected in Mk cultures (Fig. 4), confirming that the effects of PLZF on megakaryopoiesis are mediated by miR-146a.

Altogether, our results indicated that a sustained overexpression of miR-146a impaired Mk proliferation and induced retardation of Mk differentiation/maturation. This was associated with a decrease of CXCR4 expression at protein but not mRNA level, showing that normal Mk proliferation and differentiation/maturation is promoted by the PLZF mediated down-modulation of miR-146a, which causes an increased CXCR4 protein expression.

MiR-146a suppression in unilineage Mk cultures increases CXCR4 protein expression and stimulates cell proliferation and differentiation/maturation

We then evaluated whether the early suppression of miR-146a activity affects cell proliferation, differentiation and maturation in Mk cultures.

$CD34^+$ cells, seeded in unilineage Mk liquid cultures, were co-transfected after 10 hrs with: (i) antagomiR-146a oligonucleotide (α-miR-146a) or control unrelated antagomir-133a (Cont. α-miR) and (ii) a FITC-conjugated non-targeting oligonucleotide used as a tracer for FACS analysis. We sorted the FITC-positive cells by FACS and seeded them again in unilineage Mk liquid culture. We next analyzed at sequential times antagomir-146a transfected cells (α-miR-146a), as compared to control (Cont. α-miR) and untransfected cells (Control Mk) (Fig. 11). Antagomir-146a transfection induced a significant CXCR4 protein upmodulation throughout megakaryopoiesis (Fig. 11b right panel) without any significant change of CXCR4 mRNA level (left panel): this was coupled with a modest

increase in proliferation rate (Fig. 11a) and an accelerated Mk differentiation and maturation, as indicated by the increase of cells with polylobated nuclei (Fig. 11c), as well as the rise of CD61 and CD41 expression (Fig. 11d). Actually, the accelerated block of miR-146a produced a 2-3-day earlier upregulation of CXCR4 and a correspondingly earlier onset of Mk differentiation. Altogether, the results observed by transfection of antagomir-146a in Mk cells mirror those observed upon miR-146a enforced expression (Fig. 4).

Knockdown of CXCR4 in unilineage Mk cultures impairs cell proliferation and differentiation/maturation

We then blocked CXCR4 expression in Mk cultures by siRNA. Cells were cotransfected with siRNA targeting CXCR4 mRNA (CXCR4-siRNA) or nontargeting control siRNA (C-siRNA), and FITC-conjugated double-stranded RNA. FITC positive cells were then sorted and cultured. Silencing of CXCR4 mRNA (Fig. 6b) decreased CXCR4 protein level as evaluated by FACS (Fig. 6c), decreased cell proliferation (Fig. 6a) and impaired Mk differentiation/maturation, as shown by morphology (Fig. 6d) and reduced expression of the surface antigens CD61, CD9 (Fig. 6e), CD41 and CD42b (not shown), as well as by clonogenic Mk and multilineage progenitor assay (Fig. 6f).

These results are equivalent to those obtained when miR-146a or PLZF-siRNA, were transfected in Mk cultures (Fig. 4 and 5) and confirm that the crucial effects of miR-146a on megakaryopoiesis are mediated by CXCR4.

Rescue of miR-146a expression blocks CXCR4 translation and impairs PDB-induced Mk differentiation of K562-PLZF cells

To further strengthen the link between PLZF, miR-146a and CXCR4 expression, we transfected miR-146a in K562-PLZF and K562-LXSN cells, used as a model of inducible Mk differentiation[34] (Fig. 1). After co-transfection with: (a) a nontargeting scrambled oligonucleotide (C-oligo) or miR-146a and (b) a FITC-conjugated non-targeting oligonucleotide used as a tracer for FACS analysis, 85% of the cells were FITC-positive

(data not shown). FACS evaluation of CXCR4 protein expression in miR-146a transfected cells revealed that CXCR4 protein was downmodulated by miR-146a (Fig. 7a). We then analyzed the expression of Mk markers in miR-146a transfected cells after four days of PDB treatment (Fig. 7b,c): i) Mk membrane markers are clearly induced in K562-PLZF cells, as compared to LXSN cells; ii) miR-146a transfection abrogated the increase of the Mk markers CD9, CD41, CD61 expression induced by PDB; (iii) notably, in miR-146a-trasfected K562-PLZF cells the expression level of these markers was downmodulated to that observed in K562-LXSN cells. Therefore, the rescue of miR-146a expression blocks CXCR4 translation and impairs Mk-like differentiation in K562-PLZF cells, thus confirming that the effects of PLZF are exerted through the miR-146a/CXCR4 pathway.

Expression of a CXCR4 mutated in the 3' UTR rescues the inhibitory effect induced by miR-146a on Mk differentiation of K562 cells

To obtain critical evidence linking the effects of miR-146a on Mk differentiation to its activity on CXCR4 translation, we investigated the rescue of these effects upon expression of a mutated CXCR4 not targeted by miR-146a. Specifically, K562 cells induced to Mk differentiation by PDB treatment were co-transfected with both miR-146a and expression vectors for the full length CXCR4 cDNA including the 3'UTR region (wt), or a CXCR4 cDNA deleted of the entire 3'UTR, (del) or a full length CXCR4 cDNA carrying mutations of the miR-146a binding sites 2 and 3 in the 3'UTR (mut). We next measured the expression of CXCR4 and Mk differentiation markers. The results indicate that the deletion or mutation of the CXCR4 3' UTR almost abolished the effects of miR-146a on CXCR4 expression (Fig 7 d) and restored the induction of the Mk differentiation markers CD41 and CD61 (Fig 7 e, f). Thus, the inhibitory effects of miR-146a on Mk differentiation of K562 cells are rescued by CXCR4, indicating that these effects are mostly exerted through the control of CXCR4 translation.

DISCUSSION

We have identified a molecular cascade controlling normal megakaryopoiesis. This regulatory pathway involves: (a) enhanced expression of PLZF, which inhibits miR-146a transcription; (b) miR-146a downmodulation, which causes an increased translation of the target CXCR4 mRNA. In this regulatory loop miR-146a acts as an effector of the PLZF transcription factor, mediating its control activity on CXCR4 protein level and megakaryopoiesis.

MiRNAs are posttranscriptional regulators of gene expression, which play a key role in basic cell functions under normal and abnormal conditions[3,38]. Although their role in the control of normal[9] and leukemic[39] hematopoiesis has been recognized, relatively little is known so far on the specific pathways involved, particularly in megakaryopoiesis. We have previously shown that miR-221 and -222 are critical regulators of erythropoiesis through targeting of the kit receptor[11]. In addition, enhanced expression of miR-223 repressing translation of the NFI-A transcription factor is necessary for granulocytic differentiation[10]. Recently, we discovered a control network in monocytopoiesis: this pathway comprises miR-17-5p/20a/106a, which target the transcription factor AML-1, activating in turn the expression of the M-CSF receptor[12]. Here we show that miR-146a masters Mk differentiation by targeting the CXCR4 receptor. MiR-146a is involved in carcinogenesis[40] and in the regulation of the innate immune response[41]. Our results now indicate that miR-146a plays a pivotal role in the control of CXCR4 expression in Mk differentiation.

Although informatic algorithms suggest that several miRNAs might putatively target CXCR4 (see Results), their absence or specific expression profile in Mk cell lines suggests that they do not control CXCR4 protein levels in megakaryopoiesis. In contrast, we demonstrated that miR-146a interacts directly with two "seed" regions in the 3' UTR CXCR4 mRNA region, thereby inhibiting CXCR4 translation.

The CXCR4 receptor is upmodulated through all steps of the Mk differentiation pathway, from CFU-Mk through platelets (ref. 42 and our results). In normal megakaryopoiesis, chemokines, cytokines and adhesive interactions contribute to differentiation control by regulating both proliferation and the spatial relationships among precursor cells within the bone marrow microenvironment[30,31,42]. Specifically previous reports[30,31], confirmed by the hereby presented data, show that inhibition of CXCR4 blocks normal megakaryopoiesis and thrombopoiesis, indicating a major role for CXCR4 in these processes.

Our functional studies demonstrate that the upregulation of CXCR4 protein expression, triggered by the downmodulation of miR-146a, is required for Mk progenitor proliferation, as well as for Mk precursor differentiation and maturation. This conclusion is directly supported by the fact that enforced expression of miR-146a in HPCs markedly inhibits Mk colony formation, as well as initial and more advanced megakaryopoiesis in liquid suspension culture. This effect is specific for the Mk lineage, since we did not observe any inhibition of erythropoiesis or granulo-monocytopoiesis in culture assays. An opposite effect is induced by antagomir-146a transfection. In fact, the early treatment of the cells with antagomir-146a accelerated by ~ 2-3 days the upregulation of CXCR4 and produced a similar acceleration of Mk differentiation/maturation. Remarkably, the siRNA-mediated inhibition of CXCR4 protein expression in Mk culture mimics the effects of miR-146a overexpression, suggesting that CXCR4 is the major mediator of the effects of miR-146a on megakaryopoiesis. Rescue experiments with CXCR4, mutated in the 3'UTR at the level of the miR-146a target sequences or deleted of the 3' UTR, indicate that CXCR4 is the major or only mediator of the miR-146a effect on the Mk lineage. Altogether, we postulate that the miR-146a/CXCR4 pathway is a major controller of both early and late phases of megakaryopoiesis, triggering the regulated expression of the Mk-specific gene

program. This program, particularly in its late stages, may well involve the modulation of other miRNAs [43].

The molecular pathway acting through the miR-146a/CXCR4 complex is controlled by the PLZF suppressor. This transcription factor inhibits miR-146a transcription by interacting directly with a newly discovered PLZF binding site within the miR-146a promoter region. ChIP experiments, luciferase-based promoter assays and PLZF silencing studies coherently indicate that the progressive increase of PLZF expression during megakaryopoiesis represses miR-146a transcription, thereby unblocking CXCR4 protein expression and stimulating megakaryopoiesis. Here again, the effects of PLZF silencing on Mk differentiation mimic those observed upon either miR-146a overexpression or CXCR4 silencing. Furthermore, rescue experiments indicate that the PLZF action on megakaryopoiesis is essentially mediated by miR-146a.

Altogether, these studies indicate that a three-step suppressor pathway including PLZF, miR-146a and CXCR4 plays a key role in the control of megakaryopoiesis.

The PLZF/miR-146a/CXCR4 action may be in part mediated by regulation of the interactions of Mk precursor cells with other cells and, in vivo, with the extracellular matrix. In line with this hypothesis, PLZF down-regulates the adhesion molecule VLA-4 involved in the mobilization of hematopoietic and leukemic cells[32], while CXCR4 is an essential regulator of hematopoietic stem cells traffic in stem cells niches[22] and a mediator of myeloid cells homing/mobilization[28].

The CXCR4 receptor is involved in the process of cancer metastasis[44], mediates HIV entry into CD4+ lymphocytes[45] and plays a major role in the pathogenesis of rheumatoid arthritis[46]. Therefore, the discovery of inhibitors of the SDF1/CXCR4 pathway is an important objective in molecular pharmacology[47]. In view of the capacity of miRNAs and anti-miRNAs oligonucleotides to exert effective in vivo actions[48,49], our results indicate that

miR-146a may represent a key tool for the development of molecular therapy in these clinical settings.

The identification of the PLZF/miR-146a/CXCR4 cascade pathway defines a new model for the miRNA-mediated regulation of megakaryopoiesis, although we cannot exclude that PLZF and miR-146a may exert additional, minor actions through other targets. This model suggests that miRNAs act as intermediate effectors through which transcription factors exert their control on differentiation and proliferation of precursor cells. MiRNA-based regulatory mechanisms have been described in erythropoiesis[11], granulopoiesis[10] and monocytopoiesis[12]. All these control pathways target a key hematopoietic receptor: specifically, c-kit in erythropoiesis, CXCR4 in megakaryopoiesis, G-CSF receptor in granulopoiesis (our unpublished results) and M-CSF receptor in monocytopoiesis. The receptor is either a direct miRNA target, as in erythropoiesis and megakaryocytopoiesis, or is regulated by a miRNA-controlled transcription factor, as in granulocytopoiesis and monocytopoiesis. Altogether, we postulate that the cascade model depicted here may represent a general mechanism in the regulation of hematopoiesis, linking lineage(s)-specific transcription factors, miRNAs and key functional mRNA targets, particularly growth factor/chemokine receptors.

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Claims

1. Use of antisense RNA specific for all or part of the 3' untranslated region of CXCR4 protein mRNA in therapy.

2. Use according to claim 1 for the treatment of cancer.

3. Use according to claim 2 for the treatment of tumour metastasis, leukaemia and/or solid tumours.

4. Use according to claim 1 for the treatment of HIV.

5. Use according to claim 4 for prevention of HIV entry into host cells.

6. Use according to claim 1 for the treatment of Rheumatoid Arthritis.

7. Use according to claim 1 for the inhibition of megakaryopoiesis.

8. Use according to any preceding claim via CXCR4 receptor down-modulation or inhibition, especially by posttranscriptional control of CXCR4 by miR-146a.

9. Use according to any preceding claim, wherein the antisense RNA is a micro RNA having has at least 60% homology with a selected region of the 3' untranslated region of CXCR4 protein mRNA.

10. Use according to claim 9, wherein the micro RNA has the sequence of SEQ ID NO 1.

11. Use according to any preceding claim, wherein the 3' untranslated (UTR) region of human CXCR4 protein mRNA is that provided in SEQ ID NO. 5.

12. Use of an inhibitor or suppressor (antagomir) of miR-146a in therapy.

13. Use according to claim 12, for the stimulation of Megakaryopoiesis.

14. Use according to claim 12 or 13, wherein the antagomir has the sequence of SEQ ID NO 2.

15. Use of PLZF to increase CXCR4 expression or reduce miR 146a-mediated inhibition of CXCR4, in therapy.

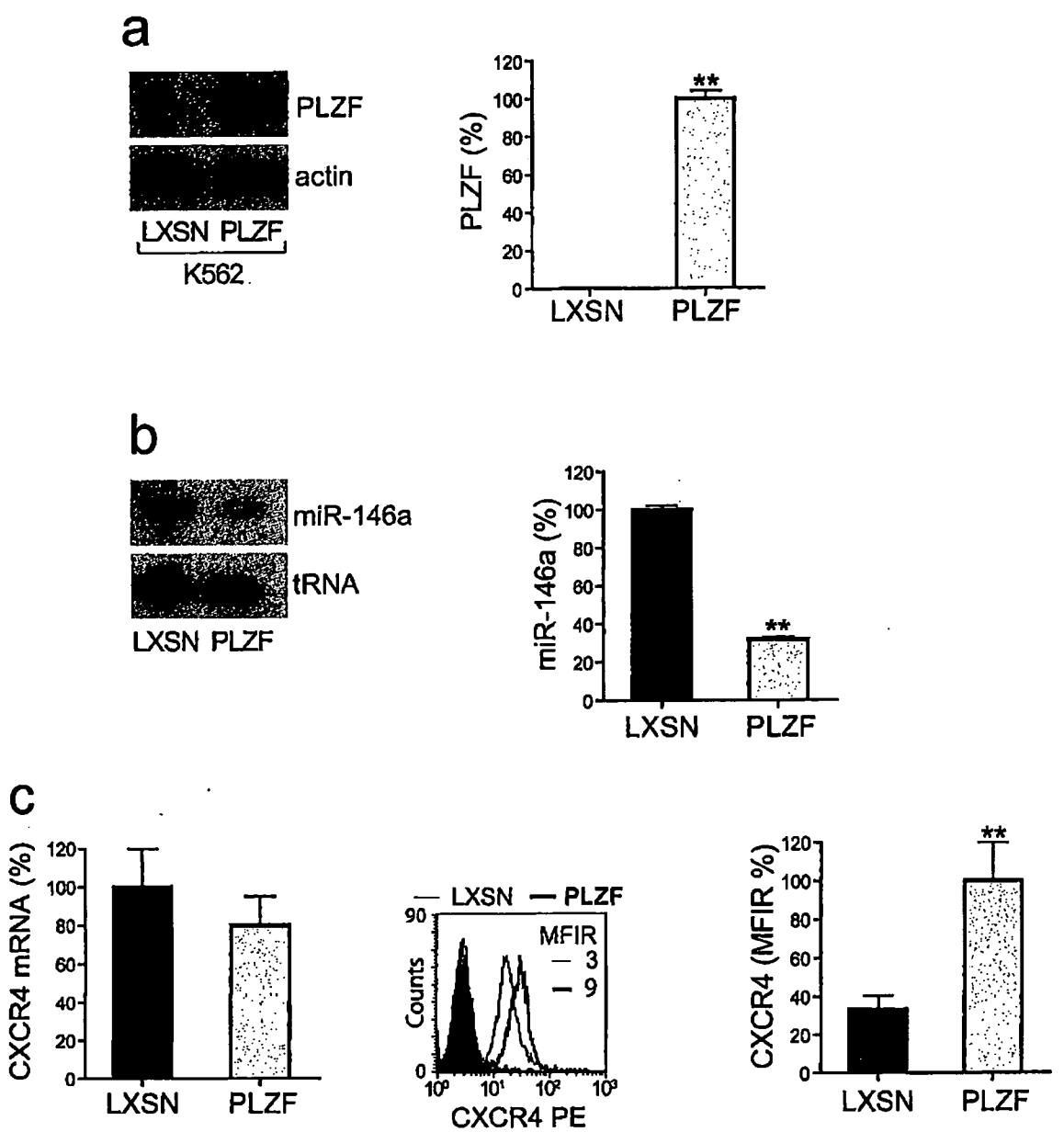
16. Use of antisense RNA specific for the mRNA sequence of PLZF to reduce CXCR4 expression or increase miR 146a-mediated inhibition of CXCR4, in therapy.

17. A vector comprising the antisense or antagomir RNA, according to any of claims 1-14 or 16, or DNA encoding said RNA.

18. A vector comprising nucleotides encoding PLZF.

19. A method of inhibiting megakaryopoiesis or treating cancer, HIV and/or Rheumatoid Arthritis, or other CXCR4-dependent tumours or disease conditions, comprising administering to a patient an antisense RNA, preferably miR-146a, and/or antisense RNA targeted to PLZF, to thereby inhibit CXCR4 expression.

20. A method of stimulating megakaryopoiesis comprising administering to a patient an antagomir of miR-146a and/or PLZF or nucleotides encoding it, to thereby increase CXCR4 expression.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LXSN PLZF
K562
PLZF (%)
120
100
80
60
40
20
0
**
LXSN
PLZF
b
miR-146a
tRNA
LXSN PLZF
miR-146a (%)
120
100
80
60
40
20
0
**
LXSN
PLZF
c
CXCR4 mRNA (%)
120
100
80
60
40
20
0
LXSN
PLZF
LXSN
PLZF
MFIR
3
9
90
Counts
0
10⁰
10¹
10²
10³
CXCR4 PE
CXCR4 (MFIR %)
120
100
80
60
40
20
0
**
LXSN
PLZF

Fig. 1a-c

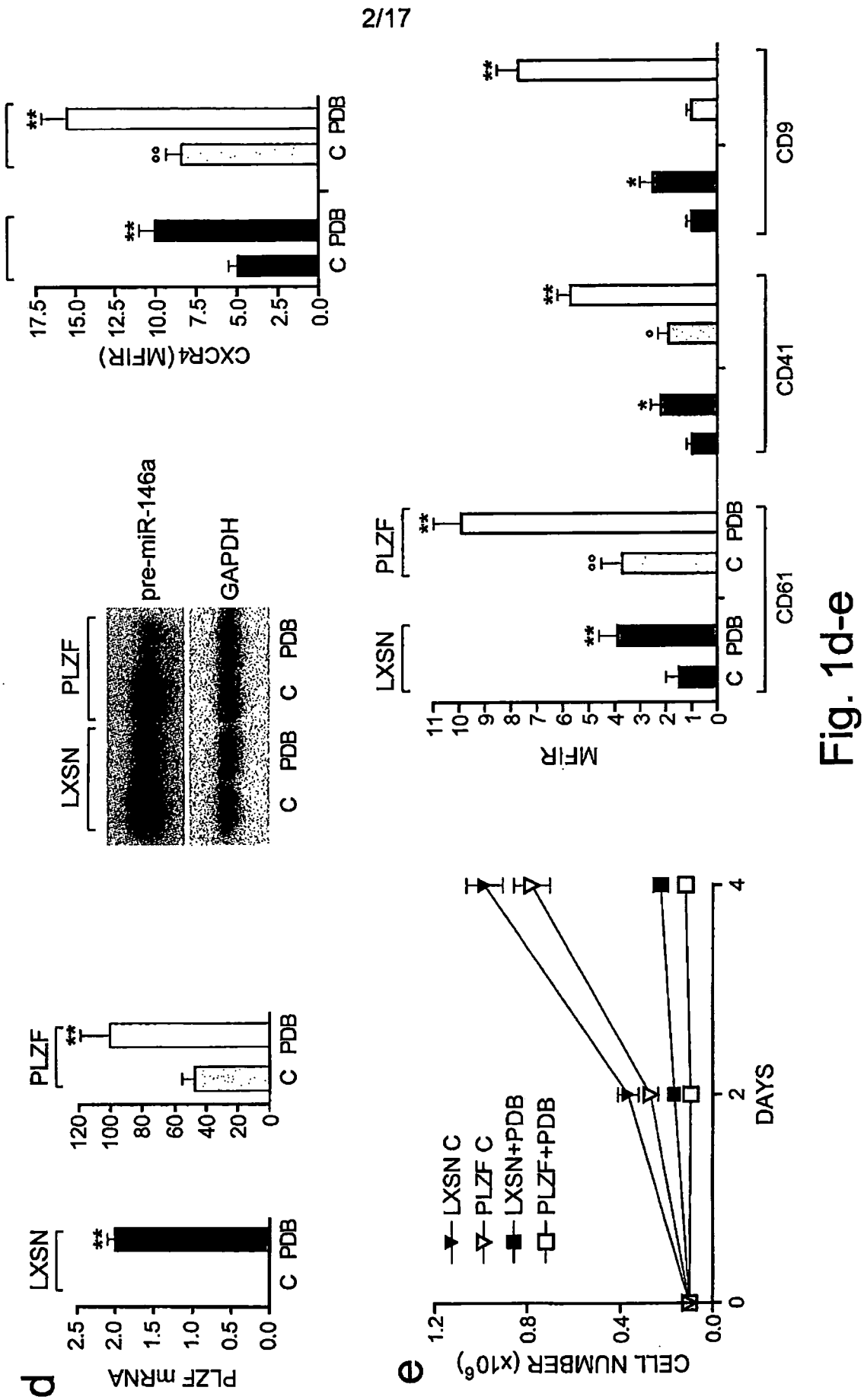

d
PLZF mRNA
LXSN
PLZF
C PDB
pre-miR-146a
GAPDH
CXCR4 (MFIR)
MFIR
CD61
CD41
CD9
e
CELL NUMBER (x10⁶)
LXSN C
PLZF C
LXSN+PDB
PLZF+PDB
DAYS

Fig. 1d-e

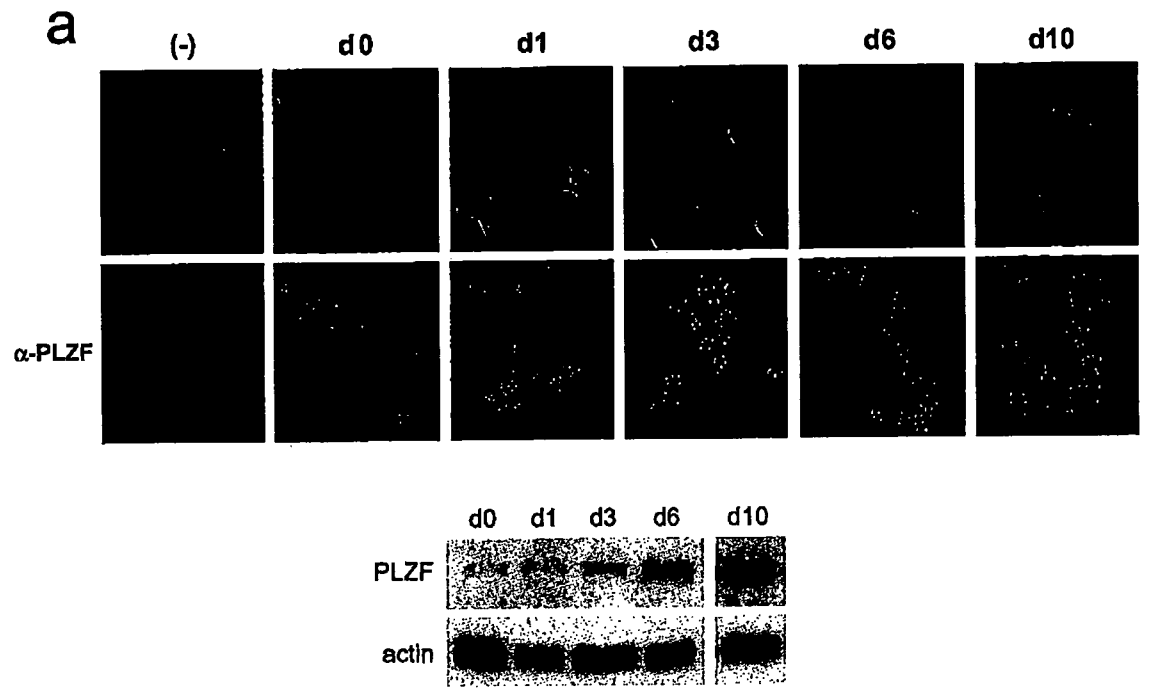

a
(-)
d0
d1
d3
d6
d10
α-PLZF
d0 d1 d3 d6 d10
PLZF
actin
b
d0 d1 d2 d3 d6
pre-miR-146a
GAPDH
miR-146a (%)
120
100
80
60
40
20
0
d0 d1 d3 d6 d8 d10
c
CXCR4 mRNA (%)
CXCR4 (MFIR %)
d0 d1 d3 d6 d8 d10

Fig. 2a-c

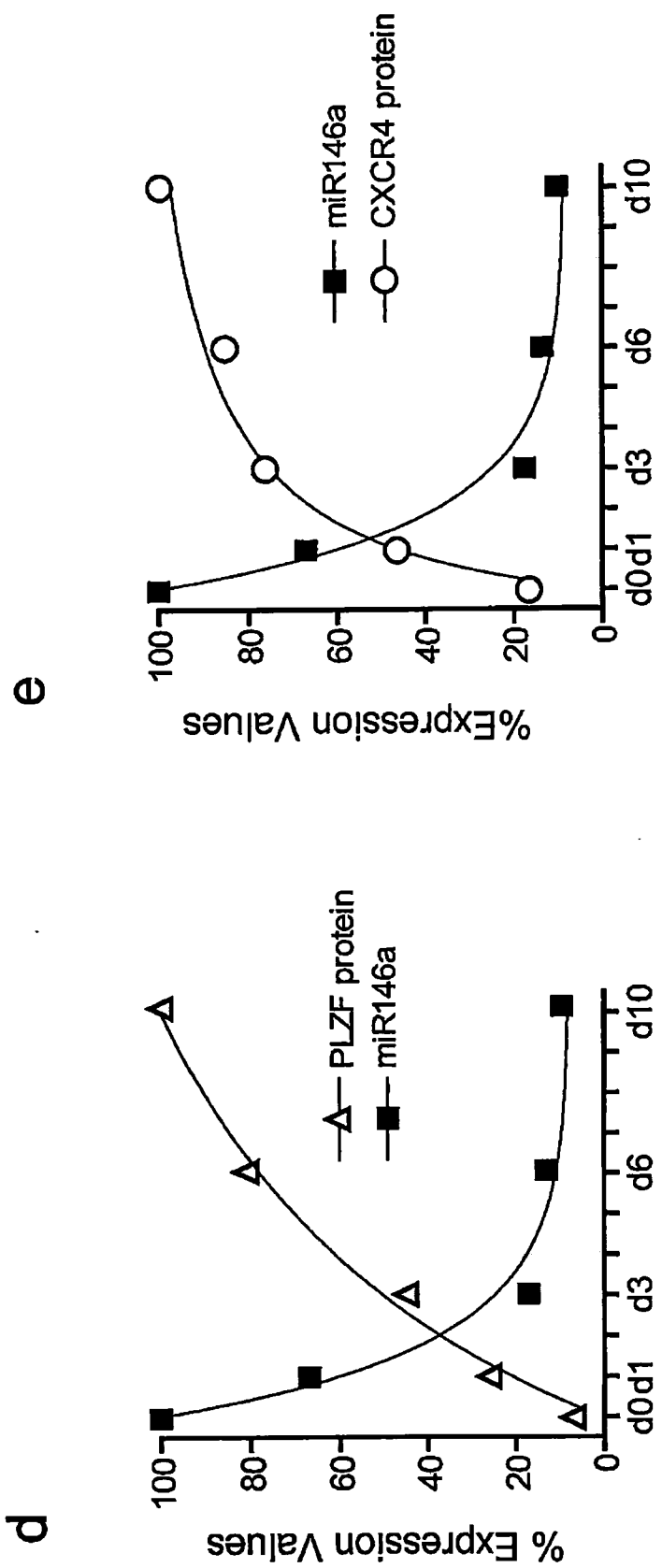

d
% Expression Values
100
80
60
40
20
0
d0 d1 d3 d6 d10
PLZF protein
miR146a
e
%Expression Values
100
80
60
40
20
0
d0 d1 d3 d6 d10
miR146a
CXCR4 protein

Fig. 2d-e

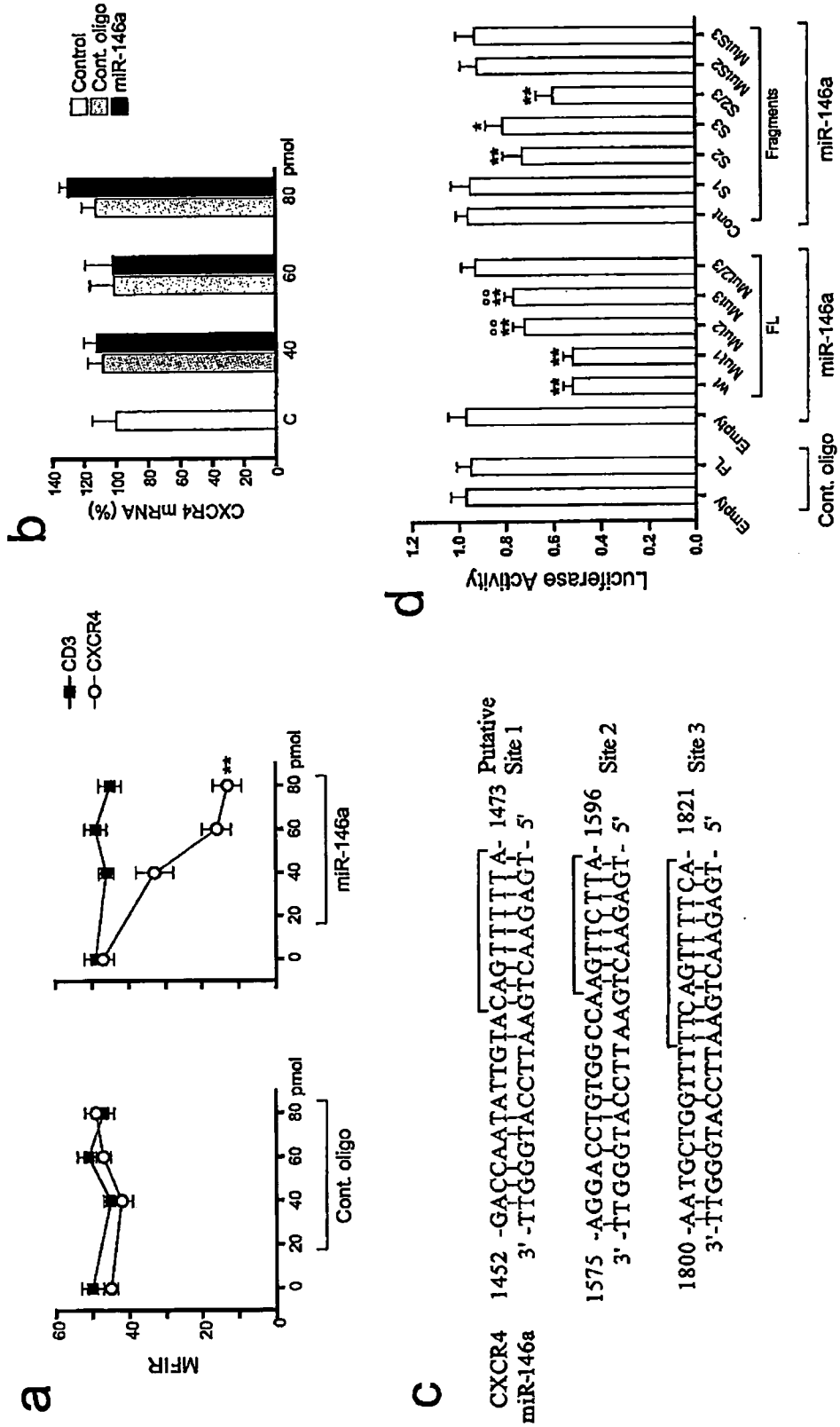
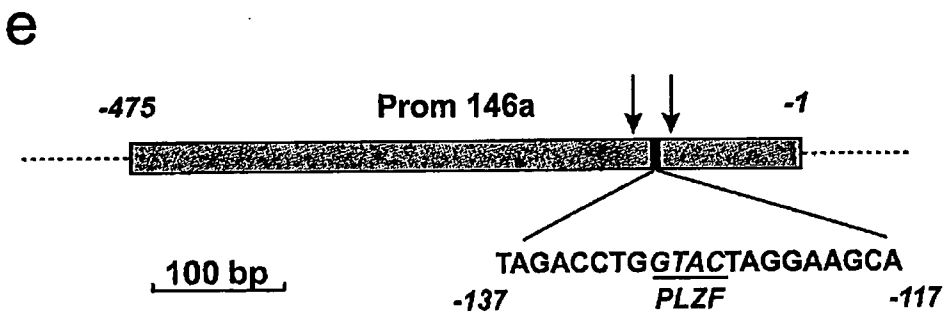
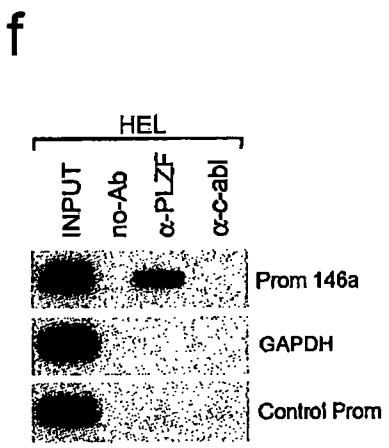
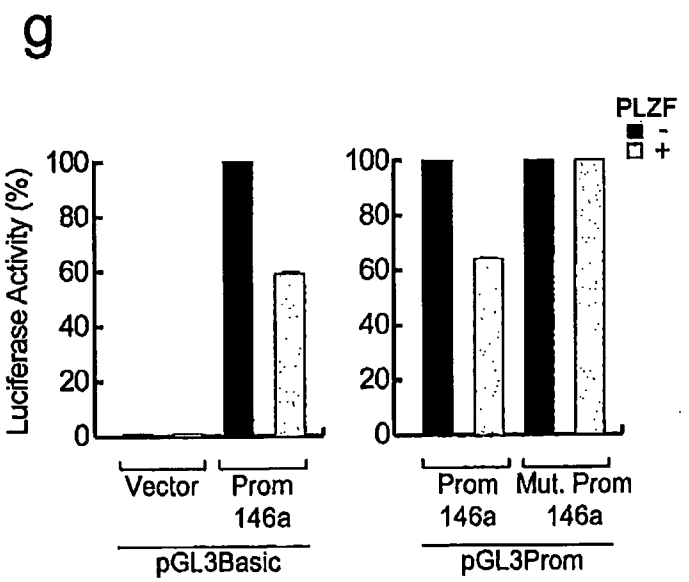

a
MFIR
CD3
CXCR4
Cont. oligo
miR-146a
b
CXCR4 mRNA (%)
Control
Cont. oligo
miR-146a
c
CXCR4 1452 -GACCAATATTGTACAGTTTTTA- 1473 Putative Site 1
miR-146a 3' -TTGGGTACCTTAAGTCAAGAGT- 5'
1575 -AGGACCTGTGGCCAAGTTCTTA- 1596 Site 2
3' -TTGGGTACCTTAAGTCAAGAGT- 5'
1800 -AATGCTGGTTTTTCAGTTTTCA- 1821 Site 3
3'-TTGGGTACCTTAAGTCAAGAGT- 5'
d
Luciferase Activity
Empty
FL
Mut1
Mut2
Mut3
Mut2/3
Cont
S1
S2
S3
S2/3
Mut S2
Mut S3
Fragments
Cont. oligo
miR-146a

Fig. 3a-d







***Mutated PLZF* binding site**
5'-TAGACCTGCGGCTAGGAAGCA-3'

Fig. 3e-g

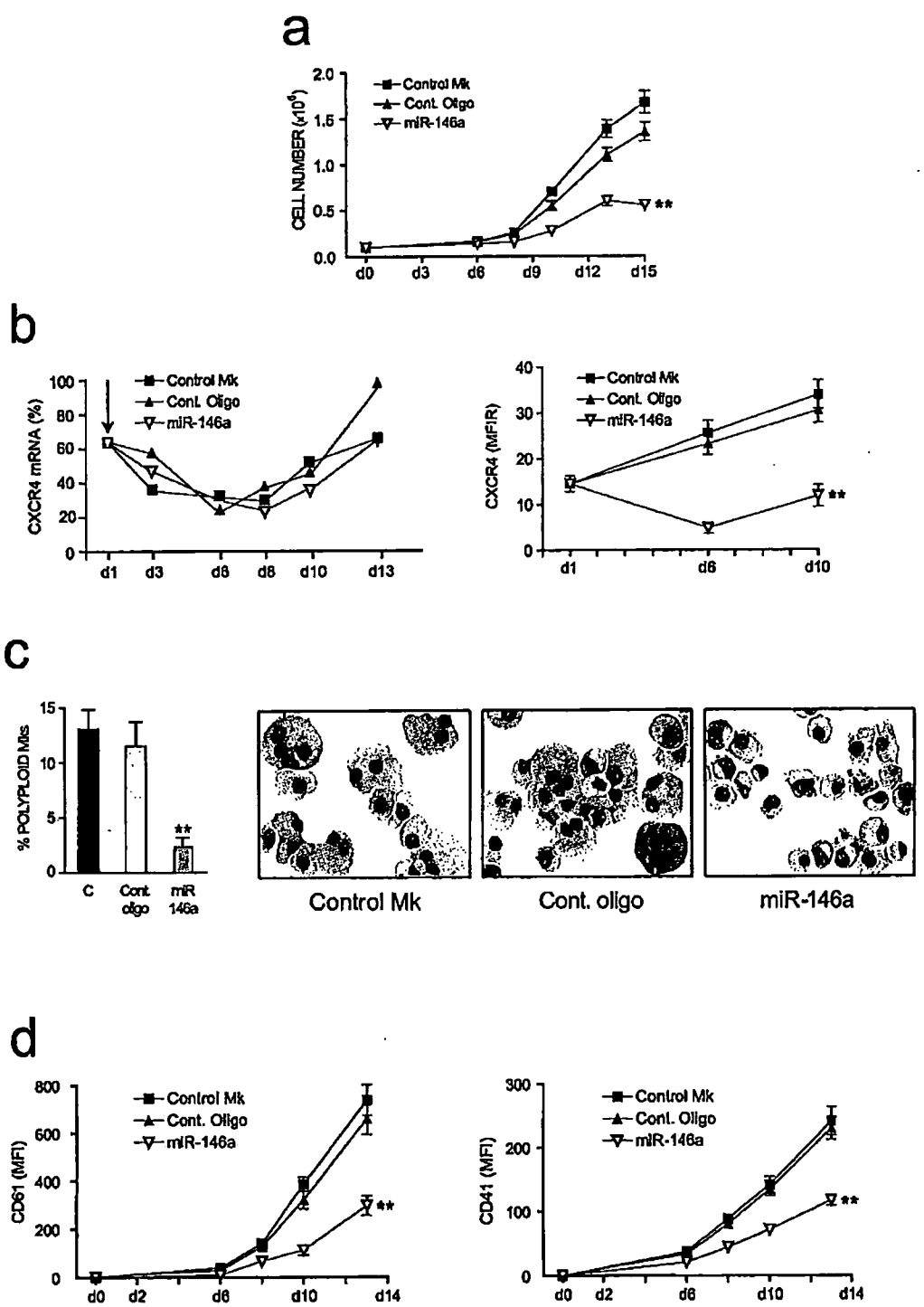

a
CELL NUMBER (x10⁶)
Control Mk
Cont. Oligo
miR-146a
d0 d3 d6 d9 d12 d15
b
CXCR4 mRNA (%)
d1 d3 d6 d8 d10 d13
CXCR4 (MFI/R)
d1 d6 d10
c
% POLYPLOID Mks
C
Cont. oligo
miR 146a
Control Mk
Cont. oligo
miR-146a
d
CD61 (MFI)
d0 d2 d6 d10 d14
CD41 (MFI)

Fig. 4a-d

e

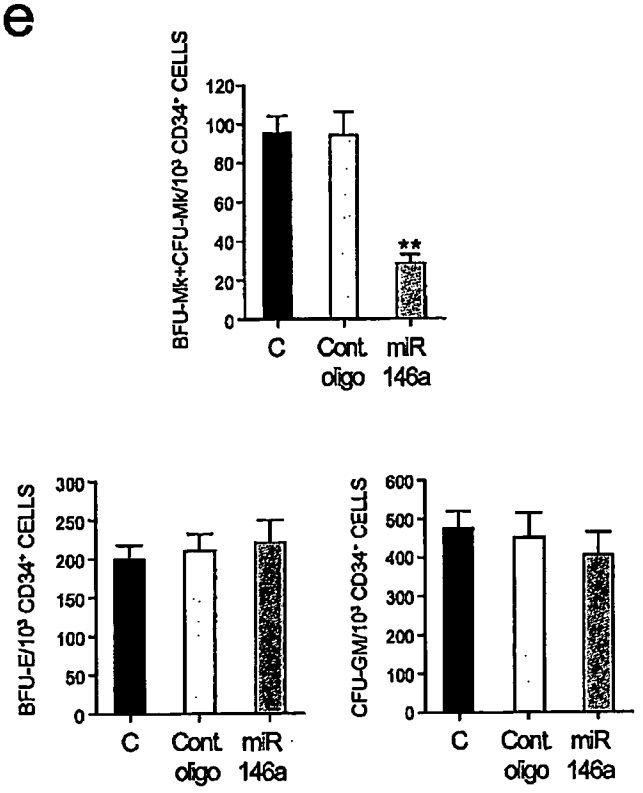

BFU-Mk+CFU-Mk/10³ CD34⁺ CELLS
120
100
80
60
40
20
0
**
C
Cont oligo
miR 146a
BFU-E/10³ CD34⁺ CELLS
300
250
200
150
100
50
0
C
Cont oligo
miR 146a
CFU-GM/10³ CD34⁺ CELLS
600
500
400
300
200
100
0
C
Cont oligo
miR 146a

Fig. 4e

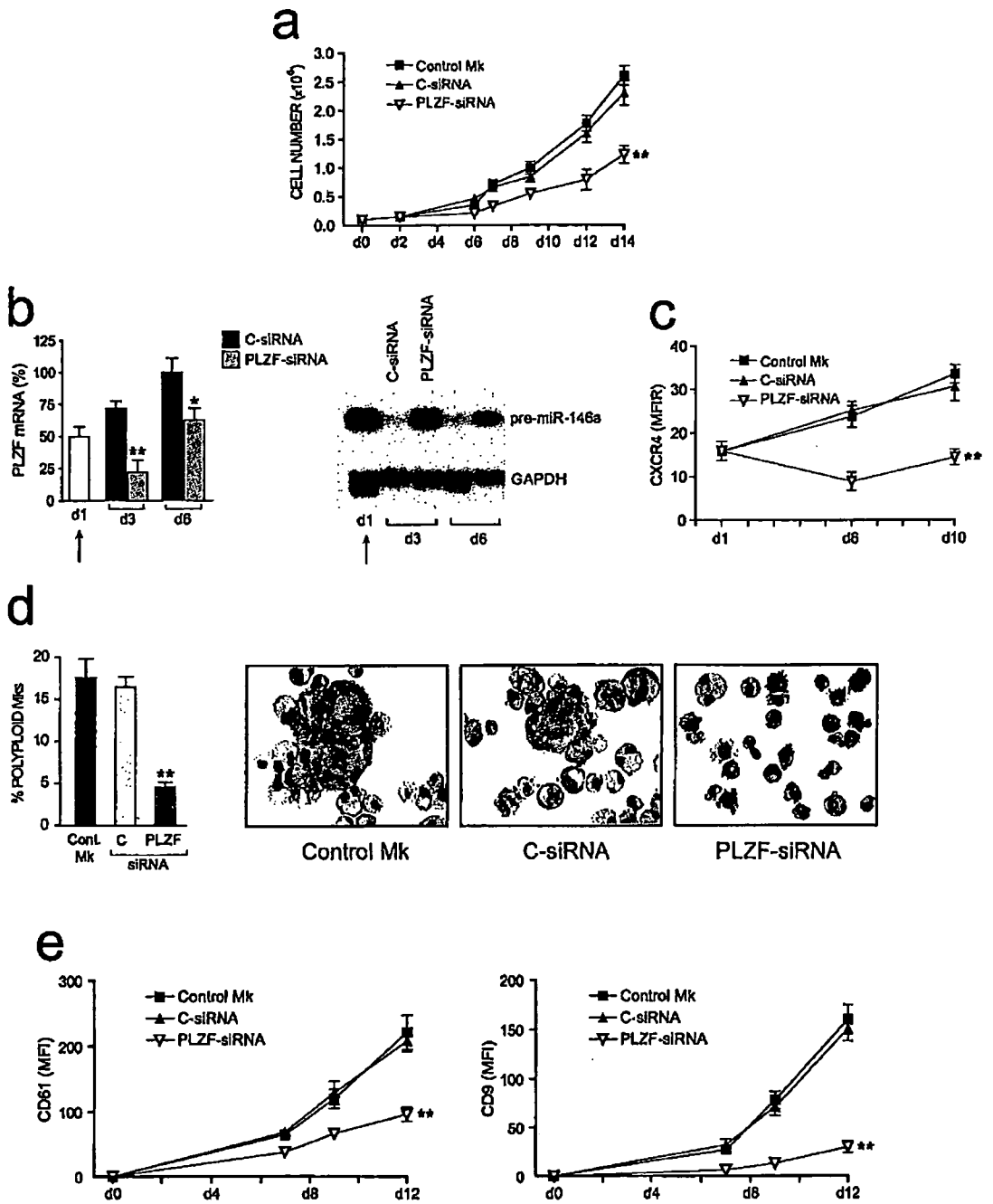

a
CELL NUMBER (x10⁶)
Control Mk
C-siRNA
PLZF-siRNA
d0 d2 d4 d6 d8 d10 d12 d14
b
PLZF mRNA (%)
C-siRNA
PLZF-siRNA
d1 d3 d6
pre-miR-146a
GAPDH
c
CXCR4 (MFIR)
Control Mk
C-siRNA
PLZF-siRNA
d1 d8 d10
d
% POLYPLOID Mks
Cont. Mk
C
PLZF siRNA
Control Mk
C-siRNA
PLZF-siRNA
e
CD61 (MFI)
CD9 (MFI)
Control Mk
C-siRNA
PLZF-siRNA
d0 d4 d8 d12

Fig. 5a-e

f

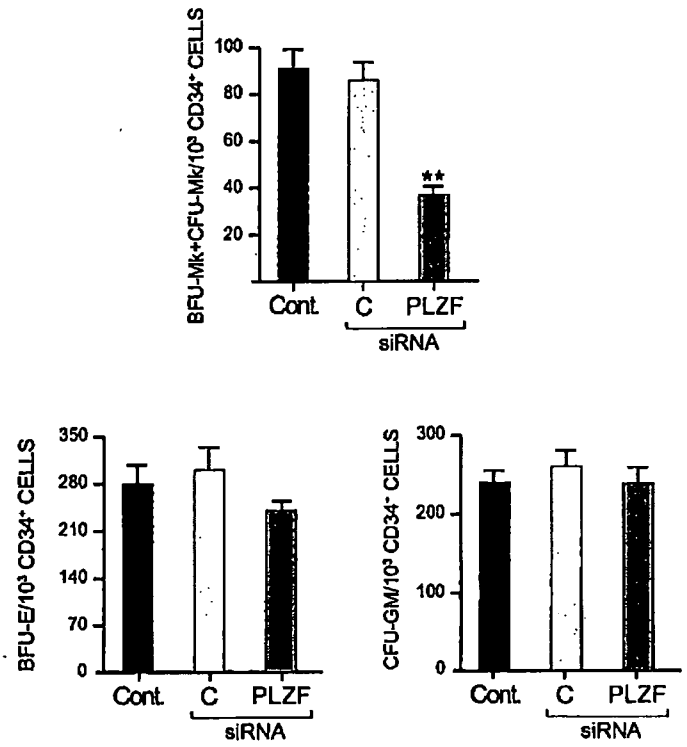

BFU-Mk+CFU-Mk/10³ CD34⁺ CELLS
100
80
60
40
20
**
Cont.
C
PLZF
siRNA
BFU-E/10³ CD34⁺ CELLS
350
280
210
140
70
0
Cont.
C
PLZF
siRNA
CFU-GM/10³ CD34⁺ CELLS
300
200
100
0
Cont.
C
PLZF
siRNA

Fig. 5f

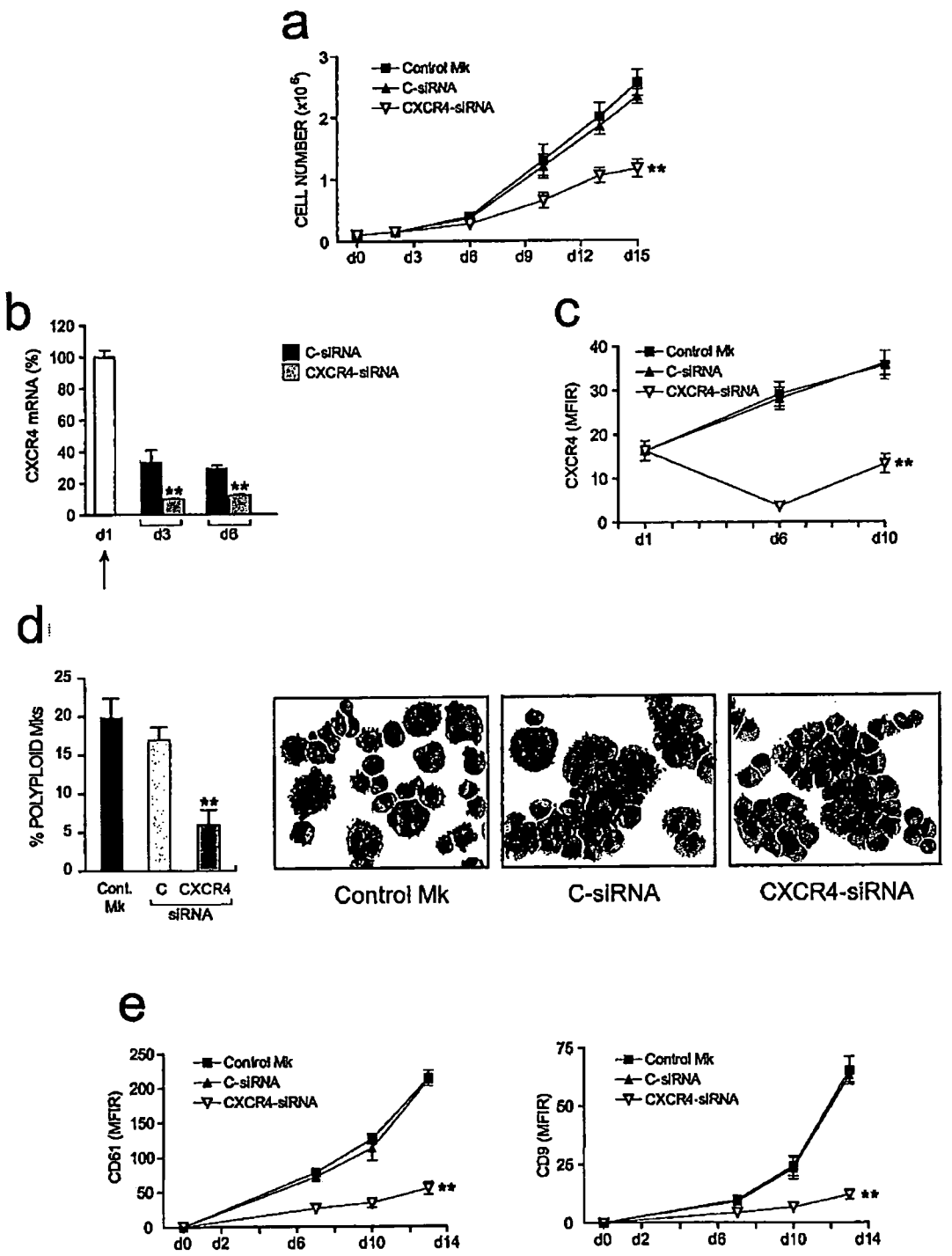

a
Control Mk
C-siRNA
CXCR4-siRNA
CELL NUMBER (x10⁶)
d0 d3 d8 d9 d12 d15
**
b
C-siRNA
CXCR4-siRNA
CXCR4 mRNA (%)
d1 d3 d6
c
Control Mk
C-siRNA
CXCR4-siRNA
CXCR4 (MFIR)
d1 d6 d10
d
% POLYPLOID Mks
Cont. Mk
C CXCR4 siRNA
Control Mk
C-siRNA
CXCR4-siRNA
e
CD61 (MFIR)
CD9 (MFIR)
d0 d2 d6 d10 d14

Fig. 6a-e

f

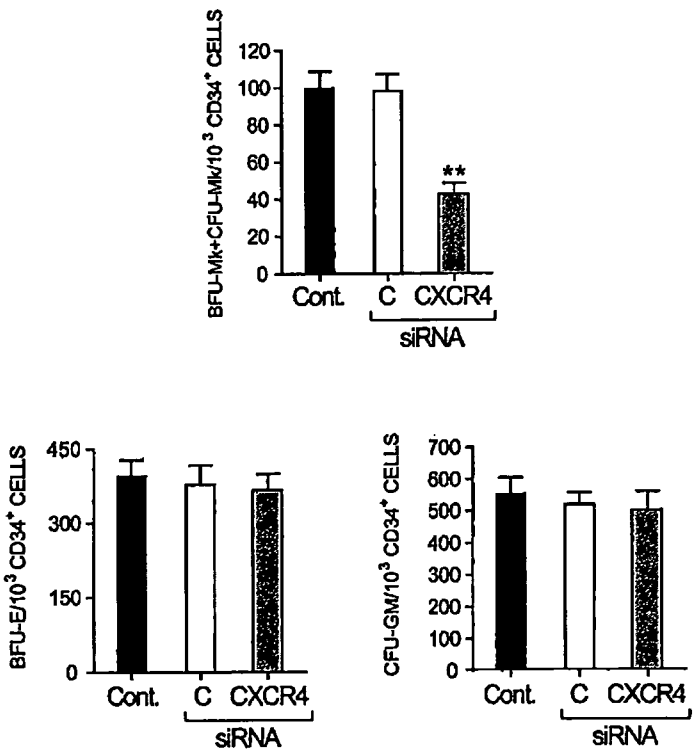

BFU-Mk+CFU-Mk/10 3 CD34+ CELLS
120
100
80
60
40
20
0
**
Cont.
C
CXCR4
siRNA
BFU-E/10^3 CD34+ CELLS
450
300
150
0
Cont.
C
CXCR4
siRNA
CFU-GM/10^3 CD34+ CELLS
700
600
500
400
300
200
100
0
Cont.
C
CXCR4
siRNA

Fig. 6f

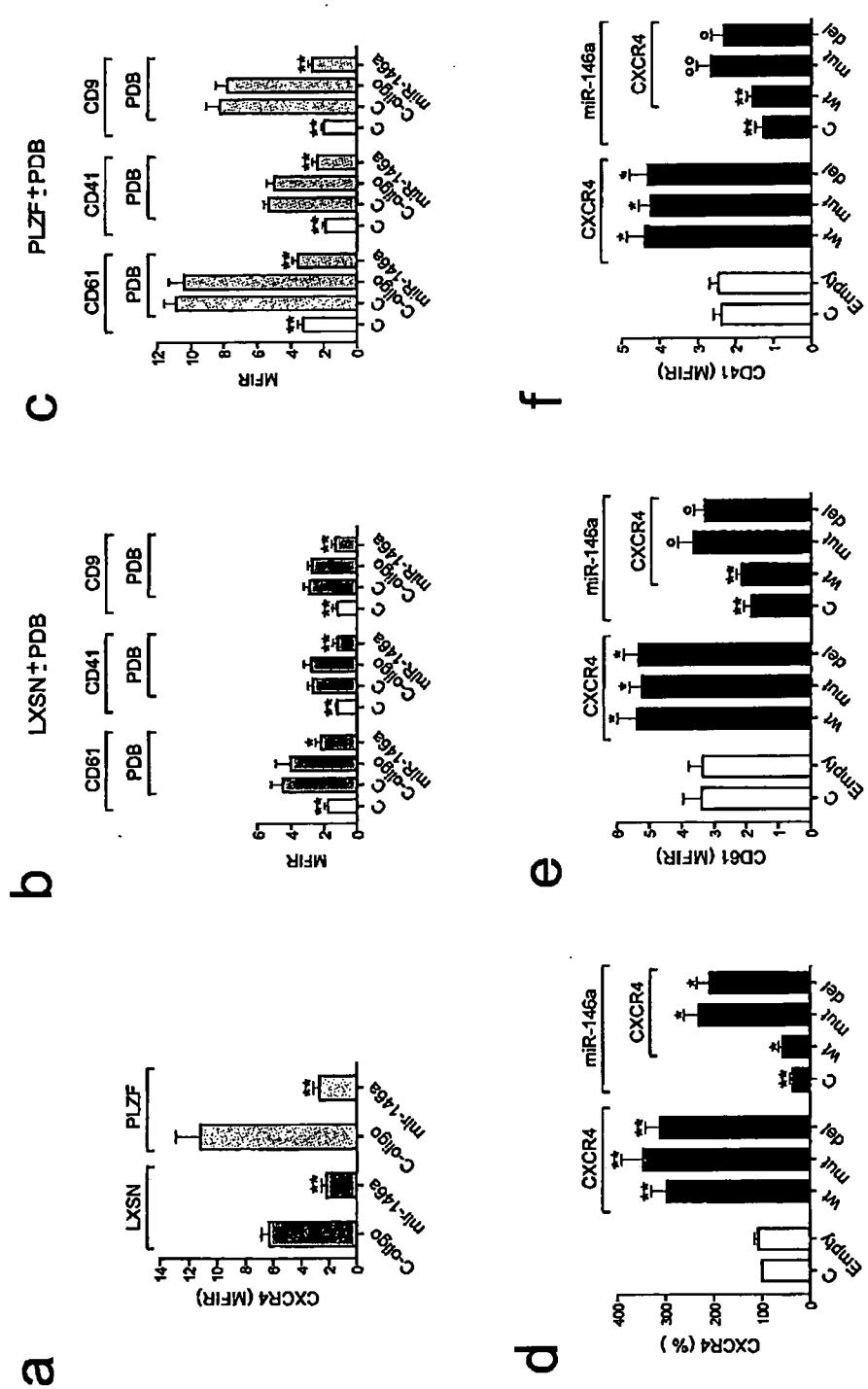

a
CXCR4 (MFIR)
LXSN
PLZF
C-oligo
mir-146a
b
LXSN+PDB
MFIR
CD61
CD41
CD9
PDB
c
PLZF+PDB
d
CXCR4 (%)
miR-146a
CXCR4
C
Empty
wt
mut
del
e
CD61 (MFIR)
f
CD41 (MFIR)

Fig. 7a-f

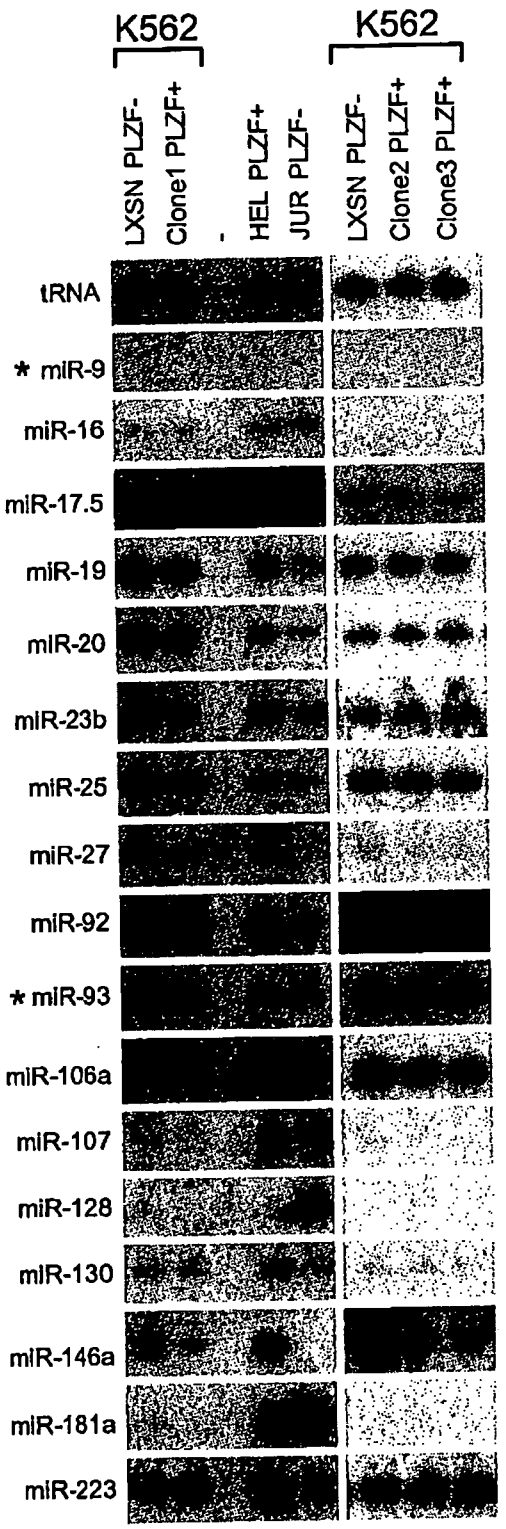

K562
K562
LXSN PLZF-
Clone1 PLZF+
-
HEL PLZF+
JUR PLZF-
LXSN PLZF-
Clone2 PLZF+
Clone3 PLZF+
tRNA
* miR-9
miR-16
miR-17.5
miR-19
miR-20
miR-23b
miR-25
miR-27
miR-92
* miR-93
miR-106a
miR-107
miR-128
miR-130
miR-146a
miR-181a
miR-223

Fig. 8

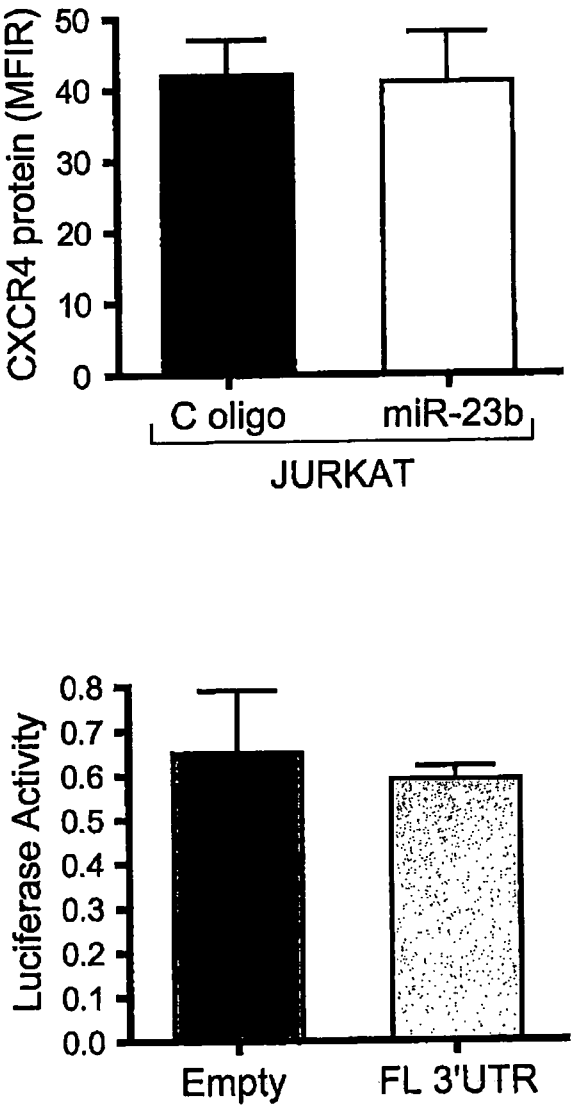

CXCR4 protein (MFIR)
50
40
30
20
10
0
C oligo
miR-23b
JURKAT
Luciferase Activity
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Empty
FL 3'UTR

Fig. 9

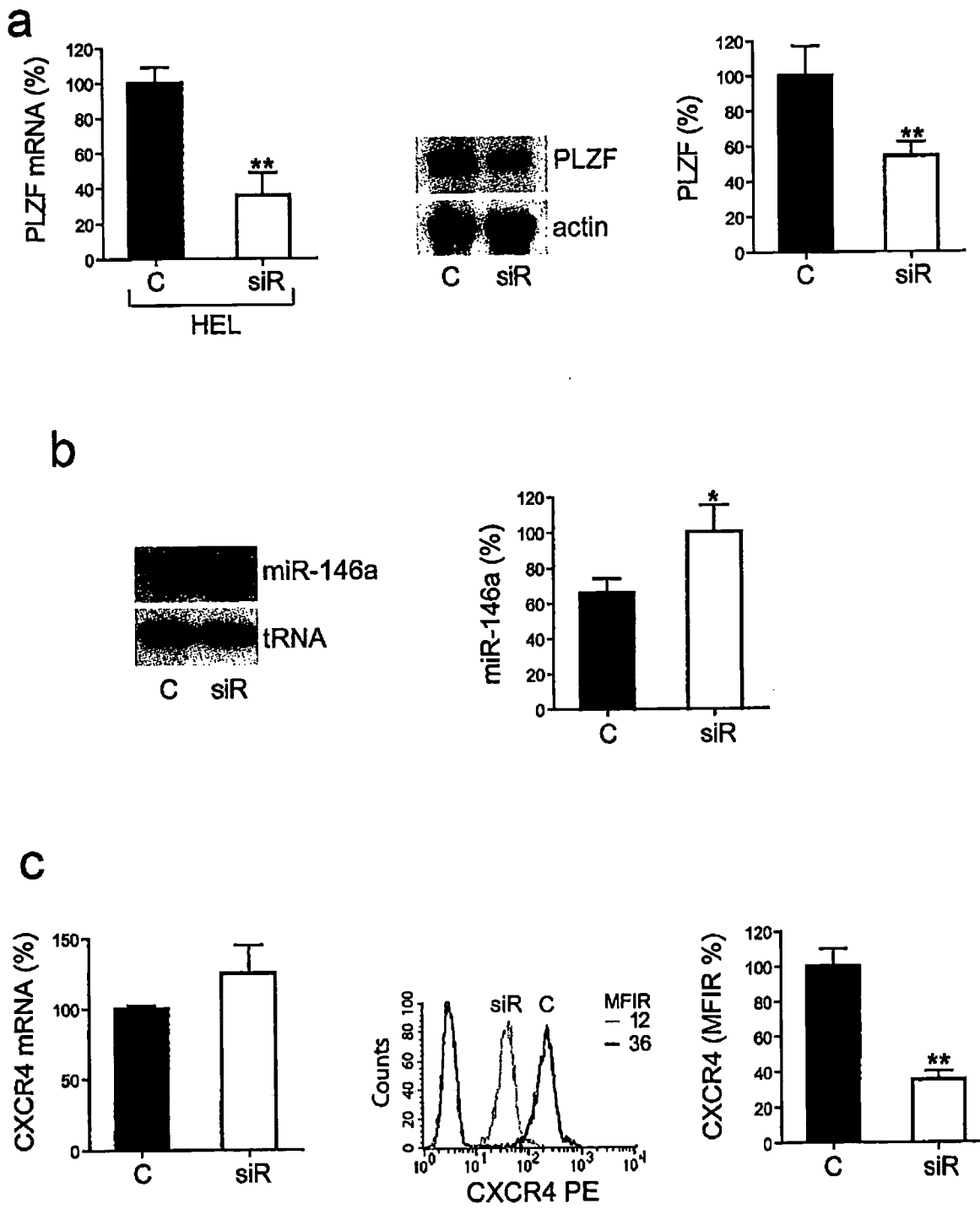

a
PLZF mRNA (%)
120
100
80
60
40
20
0
**
C
siR
HEL
PLZF
actin
C siR
PLZF (%)
120
100
80
60
40
20
0
**
C
siR
b
miR-146a
tRNA
C siR
miR-146a (%)
120
100
80
60
40
20
0
*
C
siR
c
CXCR4 mRNA (%)
150
100
50
0
C
siR
siR C
MFIR
12
36
Counts
CXCR4 PE
CXCR4 (MFIR %)
120
100
80
60
40
20
0
**
C
siR

Fig. 10a-c

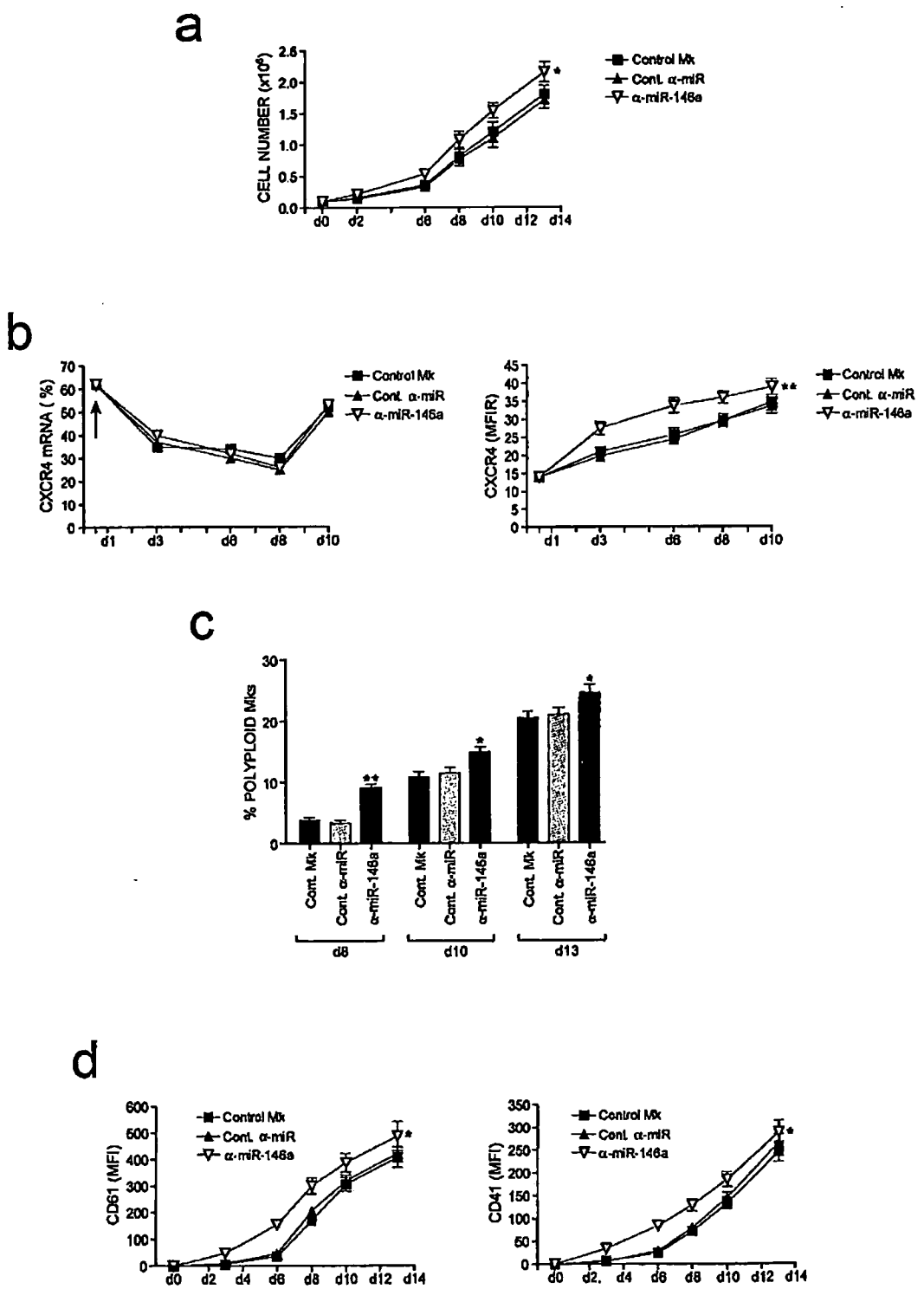

a
CELL NUMBER (x10⁶)
Control Mk
Cont. α-miR
α-miR-146a
d0 d2 d6 d8 d10 d12 d14
b
CXCR4 mRNA (%)
CXCR4 (MFIR)
d1 d3 d6 d8 d10
c
% POLYPLOID Mks
Cont. Mk
Cont. α-miR
α-miR-146a
d8
d10
d13
d
CD61 (MFI)
CD41 (MFI)

Fig. 11a-d

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/001300

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/11 A61K31/7105 A61K31/711 A61K31/713
ADD. A61P31/18 A61P35/04 A61P37/00 A61P7/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61P A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LIANG Z. ET AL.: "Blockade of invasion and metastasis of breast cancer via targeting CXCR4 with an artificial microRNA" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 363, 2007, pages 542-546, XP022288891	1-3,8,9, 11,17
X	the whole document	19
X	WO 99/50461 A (NORTHWEST BIOTHERAPEUTICS, INC. [US]; MURPHY; BOYNTON; SEHGAL) 7 October 1999 (1999-10-07)	1-3,8,9, 11,17
X	the whole document	19

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 May 2009	28/05/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Macchia, Giovanni

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/001300

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KUSUNOKI A. ET AL.: "Inhibition of the human chemokine receptor CXCR4 by antisense phosphorothioate oligodeoxyribonucleotides" FEBS LETTERS, vol. 488, 2001, pages 64-68, XP004341424	1,4,5,8, 9,11,17
X	the whole document	19
X	KUSUNOKI A. ET AL.: "Antisense Phosphorothioate oligonucleotides targeted to the human chemokine receptor CXCR4" NUCLEOSIDES, NUCLEOTIDES AND NUCLEIC ACIDS, TAYLOR & FRANCIS, PHILADELPHIA, PA, vol. 19, no. 10-12, 1 October 2000 (2000-10-01), pages 1709-1719, XP008039783 ISSN: 1525-7770	1,4,5,8, 9,11,17
X	the whole document	19
X	KUSUNOKI A. ET AL.: "Antisense oligodeoxynucleotide complementary to CXCR4 mRNA block replication of HIV-1 in COS cells" NUCLEOSIDES & NUCLEOTIDES, MARCEL DEKKER, INC, US, vol. 18, no. 6/07, 1 January 1999 (1999-01-01), pages 1705-1708, XP002926082 ISSN: 0732-8311	1,4,5,8, 9,11,17
X	the whole document	19
X	QURESHI A. ET AL.: "Gene silencing of HIV chemokine receptors using ribozymes and single-stranded antisense RNA" BIOCHEMICAL JOURNAL, vol. 394, 2006, pages 511-518, XP002526355	1,4,5,8, 9,11,17
X	the whole document	19
A	& QURESHI A. ET AL.: "Gene silencing of HIV chemokine receptors using ribozymes and single-stranded antisense RNA" BIOCHEMICAL JOURNAL, vol. 394, 2006, Retrieved from the Internet: URL:http://www.biochemj.org/bj/394/bj394051 1add.pdf> [retrieved on 2009-05-04] one page online supplementary table 1	
X	EP 1 050 583 A (HISAMITSU PHARMACEUTICAL CO. INC. [JP]; IIJIMA; GOTO; SHIMADA) 8 November 2000 (2000-11-08)	1,4,5,8, 9,11,17
X	the whole document	19

-/--

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/001300

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TAMAMURA H. ET AL.: "Identification of a CXCR4 antagonist, a T140 analog, as an anti-rheumatoid arthritis agent" FEBS LETTERS, vol. 569, 2004, pages 99-104, XP002526564 the whole document	1,6,8,19
X	HATSE S. ET AL.: "Chemokine receptor inhibition by ADM3100 is strictly confined to CXCR4" FEBS LETTERS, vol. 527, 2002, pages 255-262, XP002526565 the whole document	1,6,8,19
X	LIN S.-L. ET AL.: "Loss of mir-146a function in hormone-refractory prostate cancer" RNA, vol. 14, 3 January 2008 (2008-01-03), pages 417-424, XP002526705 Retrieved from the Internet: URL:http://rnajournal.cshlp.org/content/14/3/417> [retrieved on 2009-05-06] the whole document	1-3, 8-11,17
X	WO 2007/112754 A (SANTARIS PHAMA AS [DK]; ELMEN JOACIM [SE]; KEARNEY PHIL [AU]; KAUPPINE) 11 October 2007 (2007-10-11) claims	12
X	BERNARDO M.V. ET AL.: "Identification of apoptosis-related PLZF target genes" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 359, 2007, pages 317-322, XP022107168 the whole document	18
A	CHICARELLA S. ET AL.: "MicroRNA function in human hematopoiesis: identification of lineage-and stage-specific expression profiles, pivotal targets and regulatory circuitries" BLOOD, vol. 108, no. 11.pt.1, 16 November 2006 (2006-11-16), page 352A, XP002526356 48th Annual Meeting of the American Society of Hematology; Orlando, FL, USA; December 09 -12, 2006 the whole document	

-/--

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/001300

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YANG MING AND MATTES JOERG: "Discovery, biology and therapeutic potential of RNA interference, microRNA and antagomirs" PHARMACOLOGY & THERAPEUTICS, vol. 117, no. 1, January 2008 (2008-01), pages 94-104, XP002526727 the whole document	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2009/001300

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9950461 A	07-10-1999	AU 766675 B2	23-10-2003
		AU 3470299 A	18-10-1999
		AU 2004200560 A1	11-03-2004
		CA 2323525 A1	07-10-1999
		DE 1068357 T1	19-07-2001
		EP 1068357 A1	17-01-2001
		ES 2156581 T1	01-07-2001
		GR 2001300028 T1	29-06-2001
		JP 2002509734 T	02-04-2002
		NZ 507161 A	19-12-2003
		NZ 528767 A	26-08-2005
EP 1050583 A	08-11-2000	AU 1408300 A	13-06-2000
		WO 0031271 A1	02-06-2000
		US 6429308 B1	06-08-2002
WO 2007112754 A	11-10-2007	AU 2007234191 A1	11-10-2007
		AU 2007234192 A1	11-10-2007
		CA 2648132 A1	11-10-2007
		CA 2649045 A1	11-10-2007
		WO 2007112753 A2	11-10-2007
		EP 2007888 A2	31-12-2008
		EP 2007889 A2	31-12-2008
		KR 20080108154 A	11-12-2008