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Proje Adı (Türkçe)	Myeloid Malignansilerde Hematopoetik miRNA Ekspresyonlarının Araştırılması
Project Name (English)	Investigation of miRNA Ekspressions in Myeloid Hematopoietic Malignancies

PROJE ÖZETİ(TÜRKÇE)

MikroRNA'lar genom üzerinde protein kodlayan intron veya ekzon bölgeleri ve protein kodlamayan bölgelerdeki RNA genlerinden transkripsiyonu sağlanan, fakat proteine translasyonu gerçekleşmeyen, fonksiyonel RNA molekülleridir. Eskiden fonksiyon görmediği düşünülen bu küçük moleküllerin günümüzde hücre diferansiyasyonu, proliferasyonu ve apopitoziste önemli kritik roller üstlendiği anlaşılmıştır. Hematopoezde mikro- RNA'ların rolünü anlamak için bir çok çalışma yapılmış ve bu küçük moleküllerin hematopoezde farklılaşma, çoğalma ve apoptoz gibi çok önemli hücresel olaylarda kritik öneme sahip olduğu gösterilmiştir. miRNA'ların bu önemli hematopoetik rolüne parelel olarak malign hematolojik hastalıkların etiyolojisinde yer almaları kaçınılmazdır. Kanser gelişiminde mikroRNA'lar hedefledikleri mRNA'lara bağlı olarak tümör süpresör ve onkogen olarak fonksiyon gösterebilirler. Bulundukları şartlara bağlı olarak bazı mikroRNA'lar ise hem tümör süpresör hemde onkogen karakter gösterebilirler. Mikro-RNA'ların özellikle kanserin erken tanı, tedavi ve prognozunun belirlenmesinde, kanserli dokulardaki varlığı, ekspresyon paternindeki değişiklikleri ve hedefledikleri mRNA'ların saptanması ile önemli sonuçlar sağlanacağı gerçeği ortaya çıkmıştır. Bu konuda yapılan çok sayıda çalışma ile bazı mikro-RNA'ların doku ve hastalık türü için spesifik sayılabilecek özellikte olduğu gösterilmiştir. Normal ve patolojik dokular arasında farklı seviyede ifade edilen miRNA'lar tespit edilerek, insan kanserlerinde tanı, tedavi ve prognozun belirlenebilmesinde de yararlı olacağı kesindir. Bu çalışmada son dönemde hem normal hematapoezde, hem de maling hastalıklarda oldukça fazla sayıda araştırılan miRNA ekspresyonları, myeloid malignansi hastalarında araştırılacaktır. Myeloid malignansilerle ilişkili miRNA ekspresyon paternlerinin saptanması, myeloid malignansi etiyogenezinin aydınlatılmasına ve gelecekte bu alanda yeni ilaç rejimlerinin geliştirilmesine ön ayak olacaktır. Ayrıca bu çalışmada araştırılacak olan miRNA'ların ekspresyonları myeloid malignansilerin tedavisinde kullanılacak ilaç kaynaklarının saptanabilmesine, hastalık ilerlemesinde ve lösemik transformasyonlardaki rollerini göstererek hastalık patogenezini daha iyi anlamamamıza ve daha yeni miRNA tabanlı tedavilerin gelişmesine yol açabileceğini, aynı zamanda da hastalık özgül miRNA'ların myeloprolifatif hastalıkların ayırıcı tanısında kullanılabileceğini düşünmekteyiz.

PROJECT ABSTRACT (ENGLISH)

MicroRNAs are encoded within protein coding genes and in both introns and exons of mRNA liken on-coding RNAs. MicroRNAs are functional RNA molecules which code protein on genom, intorns or exons areas and the transcription of RNA genes at the non-coding area, but not ocur transcription to protein. These small molecules are thought as non-function molecules in the past, but today it is understood that they have critical roles in cell differentiation, proliferation, and apoptosis. Ever since the first use of microRNA term, the substantial researches have been done to understand the role of micro- RNAs at the normal and malignan hematopoiesis and considerable development are occured by the result of these researches. As a result of these researches, small molecules have been found to having critical importance on cellular structures such as differentiation, proliferation and apoptosis (programmed cell death). Our working group is 50 patients who got myeloid malignancies diagnosis or preminary diagnosis at Hematology Department at M.Ü. Faculty of Medicine Research and Training Hospital between the years 2016-2017. Control group will consist of healthy volunteers. Genetic analizes samples from the bone marrow of the patients will be carried out the school of medicine departmant of medical biology laboratory in Mersin University. Infromed consent form will be signed to the participants both experiment and control groups. MicroRNAs may act as oncogenes ant tumor supressors depending on the mRNAs on their target. Depending on the circumstances in which they, some microRNAs may show both tumor supressor and oncogenes. The MicroRNA existance of parenchyma, chance at the experssion patterns and the determination of targeted mRNAs provide important results especially in the early diagnosis of cancer, treatment of cancer and determination of prognosis. A large number of studies on this subject have shown that some microRNAs are spesific for tissue and property. This rapid development process, particularly at the gene

theraphy will come with significant genetic changes. It is obvious that the determination of the different level of MicroRNAs at the normal and pathological tissues will be benefical at treatment of cancer and determination of prognosis. In this study, the role of the MiRNA expression which are investigated both normal hematopoiesis and maling disease resently, on myeloid malignancies will be investigated. The determination of miRNA expression patterns related to myeloid malignancies will be initiative for clarification of myeloid malignancy etiyohene and development of new medicine market at this field. Furthermore, the miRNA expression which is studied at this research may help to the determination of the source of medicine, disparate impact of the different myleoid malignancies on hematopoiesis. It may also help to determine the role at the progression of the disease and leukemic transformation which helps to understand better to the disease pathogenesis and development of new miRNA based treatment. In addition to these we may use the disease specific miRNAS at definitive diagnosis of the myeloprolifative disease.

ANAHTAR KELİMELER(TÜRKÇE)

1. Hematopoez	**2.** mikroRNA	**3.** myeloid malignansi	**4.** Kemik iliği	**5.** Ekspresyon	**6.** Reel time-PCR

KEYWORDS (ENGLISH)

1. Hematopoiesis	**2.** microRNA	**3.** myeloid malignancies	**4.** Bone marrow	**5.** Expression	**6.** Reel time-PCR

PROJE YÜRÜTÜCÜSÜ

Adı	MUSTAFA ERTAN	**Telefon**	5368108848
Soyadı	AY	**Faks**	
T.C. Kimlik No	22393888362	**Birimi**	TIP FAKÜLTESİ
e-Mail	ertanay@mersin.edu.tr	**Bölümü**	TEMEL TIP BİLİMLERİ

YÜKSEK LISANS ÖĞRENCISI

Adı	CEMİLE	**Telefon**	5547294876
Soyadı	ERSÖZ	**Faks**	
T.C. Kimlik No	24946560084	**Birimi**	TIP FAKÜLTESİ
e-Mail	ylbio201507@mersin.edu.tr	**Bölümü**	TEMEL TIP BİLİMLERİ BÖLÜMÜ

PROJENİN AMACI

Kanser gelişiminde mikroRNA'lar (miRNA) hedefledikleri mRNA'lara bağlı olarak tümör süpresör ve onkogen olarak fonksiyon gösterebilirler. Bulundukları şartlara bağlı olarak bazı miRNA'lar ise hem tümör süpresör hemde onkogen karakter gösterebilirler. miRNA'ların özellikle kanserin erken tanı, tedavi ve prognozunun belirlenmesinde, kanserli dokulardaki varlığı, ekspresyon paternindeki değişiklikleri ve hedefledikleri mRNA'ların saptanması ile önemli sonuçlar sağlayacağı gerçeğini ortaya çıkarmıştır. Bu konuda yapılan çok sayıda çalışma ile bazı miRNA'ların doku ve hastalık türü için spesifik sayılabilecek özellikte olduğunu göstermiştir. Bu genetik hızlı gelişim sürecinde gen tedavisi başta olmak üzere yeni tedaviler önemli değişiklikleri beraberinde getirecektir. Normal ve patolojik dokular arasında farklı seviyede ifade edilen miRNA'lar tespit edilerek, insan kanserlerinde tanı, tedavi ve prognozun belirlenebilmesinde de yararlı olacağı kesindir. Bu çalışmada son dönemde hem normal hematopoezde, hem de malign hastalıklarda oldukça fazla sayıda araştırılan miRNA ekspresyonlarının, myeloid malignansilerdeki rolleri araştırılacaktır. Myeloid malignansilerle ilişkili miRNA ekspresyon paternlerinin saptanması, myeloid malignansi etiyogenezinin aydınlatılmasına ve gelecekte bu alanda yeni ilaç rejimlerinin geliştirilmesine ön ayak olacaktır.

PROJENİN ÖNEMİ

Normal ve maling hematopoezdeki miRNA ekspresyonları arasındaki ilişkiyi saptamak için giderek artan sayıda çalışma yapılmakla birlikte eldeki verilerle şu anda hematolojik malignansilerde miRNA'nın direkt rolünü gösteren çok az sayıda veri vardır. Bunun birinci nedeni miRNA'ların çok sayıda farklı hedeflerinin olmasının yanı sıra miRNA'ların eşlik ettiği çok fazla sayıda karmaşık ve birbiri ile örtüşen hücresel yolakların olmasıdır. Hematopoezde görevli birçok miRNA tanımlanmıştır. Çalışmamızda hematopoezde görevli miRNA'lardan; miR-155,

miR181a, miR-221, miR-222, miR-223, miR-451 ekspresyonlarının myeloid malignansi hastalarının kemik iliği hücrelerindeki değişimi araştırılacaktır. Elde edilecek verilerin myeloid malignansi gelişim sürecindeki etkileri tartışılacaktır. Eskiden fonksiyon görmediği düşünülen bu küçük moleküllerin günümüzde hücre diferansiyasyonu, proliferasyonu ve apopitoziste(programlı hücre ölümü) önemli kritik roller üstlendiği anlaşılmıştır. MikroRNA terimi ilk kullanıldığı günden beri normal ve malign hematopoezde miRNA'ların rolünü anlamak için büyük çapta çalışmalar yapılmış ve sonucunda önemli gelişme kaydedilmiştir. Yapılan çalışmalar sonucunda bu küçük moleküllerin hematopoezde farklılaşma, çoğalma ve apoptoz gibi çok önemli hücresel olaylarda kritik öneme sahip olduğu gösterilmiştir.

MikroRNA'ların kanserleşme sürecine etkisi olduğu ilk olarak 2001 yılında miR-15a ve miR16-1'in keşfedilmesi ile rapor edildi. Ancak bu miRNA'ların kanserleşme sürecine etki mekanizmaları 2005 yılında Cimmino ve arkadaşlarının KLL (Kronik Lenfositik Lösemi) bireylerde yaptıkları çalışma ile ortaya konuldu. Çalışma sonucunda adı geçen iki miRNA'nın ekspresyon seviyelerinin KLL hücrelerinde, anti-apoptotik B hücreli lenfoma proteini olan Bcl-2'nin üretimi ile ters ilişkili olduğu tespit edildi. Bununla birlikte tümör süpresör miRNA'ların tersine, onkogenik miRNA'lar çoğunlukla kanser türlerinde kontrolsüz büyümeyi arttırıcı ve/veya antiapoptotik yönde fonksiyon gösterirler. Onkoprotein kodlayan mRNA'ları hedefleyerek etki gösterir ve miRNA'ların düzeyi düşünce, hücre yaşam süresini uzatan ve proliferasyonu artıran onkoproteinlerin artmasına neden olabilir.

Hematolojik karsinogenez oldukça karmaşık bir süreç olmakla birlikte genel olarak anormal hematopoez ile ilşkilidir. Önerilen bu araştırmada da olduğu gibi anormal hematopoezden sorumlu hücresel değişimlerin belirlenmesi, hastalık etiyopatogenezinin aydınlatılması yanı sıra yeni tanı ve tedavi protokollerinin oluşturulmasına katkı sağlayacaktır.

KONU, KAPSAM VE LİTERATÜR ÖZETİ

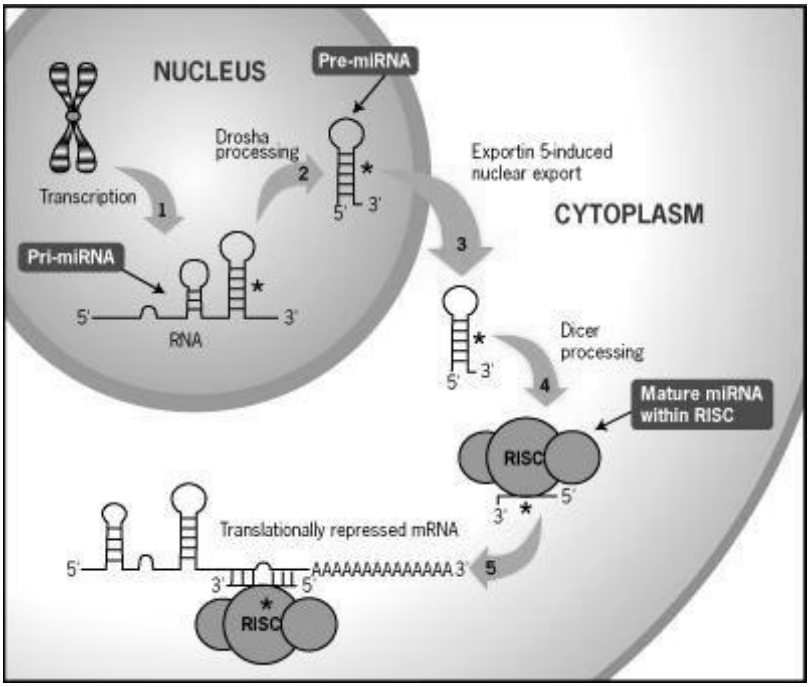
Hematolojik malignansiler, kan, kemik iliği, lenfoid nod ve lenfatik sistemin diğer kısımlarını etkileyen malignant neoplazmlardır ve genellikle 3 grupta incelenirler: Lösemi, Lenfoma ve Multipl Myelom. Bu malignensiler, miyeloid ve lenfoid olmak üzere 2 farklı hücre serisinden köken alırlar (1). Lenfotik lösemi ve miyelomlar, lenfotik hücre hattından köken almakla birlikte Akut Miyeloid Lösemi (AML), Kronik Miyeloid Lösemi (KML) ve myelodisplastik sendromlar ise miyeloid hücrelerden köken alırlar. Bu malignansilerin prevalansı batı ülkelerine kıyasla Asya ve Avrupa ülkelerinde daha düşük iken, düşük ve orta gelirli ülkelerde insidans giderek artmaktadır. Hematolojik malignensiler, yüksek morbidite ve mortaliteye yol açtığından erken tanı ve etkili tedavi oldukça önemlidir. Her ne kadar hematolojik malignensilerin biyolojisini anlamakta ve yeni tedavi yöntemlerinin gelişiminde yol katedilmiş olsa da, hala çok sayıda bilgi ve çalışmaya ihtiyaç duyulmaktadır. Tek bir molekülü hedef alan tedavi yöntemleri (KML dışında) istenilen sonuçları vermemekle birlikte hastaların büyük kısmı hastalığın tekrar nüksetmesi sonucu hayatını kaybetmektedir. Hastalığın etiyolojisini anlamamızı güçleştiren nedenler arasında; yeni hedef genlerin henüz tanımlanamamış olması, genetik ve epigenetik değişimlerdeki karmaşıklık ve kilit sinyal yolaklarındaki moleküllerin eksikliği ya da fazlalığı yer almaktadır (2).

MikroRNA'lar genom üzerinde protein kodlayan intron veya ekzon bölgeleri ve protein kodlamayan bölgelerdeki RNA genlerinden transkripsiyonu sağlanan, fakat proteine translasyonu gerçekleşmeyen, fonksiyonel RNA molekülleridir. İnsan genomunda miRNA'ları kodlayan yüksek seviyede korunmuş yüzlerce gen bölgesi keşfedilmiştir. Şu an itibariyle, insan genomunda 1000'in üzerinde mikroRNA tanımlanmıştır. İlk mikroRNA, Lee ve çalışma arkadaşları tarafından 1993 yılında Victor Ambros laboratuarında yuvarlak solucan olan Caenorhabditis elegans'ta lin-4 olarak adlandırdıkları genin hiçbir protein kodlamamasına karşın 22 nükleotid uzunluğunda küçük bir RNA transkribe etmesiyle rapor edildi. Ancak bulunan bu genetik materyal için mikroRNA terimi ilk defa 2001 yılından itibaren kullanılmaya başlanmıştır (3).

MikroRNA'lar birbirini izleyen üç basamaklı işlem sonucunda meydana gelir. İlk basamakta miRNA genlerinden primer miRNA (pri-miRNA)'ların transkripsiyonu gerçekleşir. İkinci basamakta primiRNA'lar prekürsör miRNA (pre-miRNA)'lara nükleus içinde dönüştürülür. Üçüncü ve son basamakta olgun miRNA'larin sitoplazma içinde oluşumu gerçekleşir (Şekil1)

MikroRNA'lar, primer transkript (pri-miRNA) olarak RNA polimeraz II enzimi tarafından genomik DNA'dan sentezlenir. Pri-miRNA (500-3000 baz), "cap" ve "poli A" kuyruğuna sahip sap-ilmik yapısındadır. Çekirdekte pri-miRNA, RNAaz III enzim ailesinin bir endonükleazı olan Drosha ve kofaktörü Pasha (veya DGCR8), tarafindan yaklaşık olarak 70 nükleotid uzunlugunda olan pre-miRNA'ya dönüştürülür. Bir nükleaz olan Drosha ile çift iplikli RNA bağlayıcı bir protein olan Pasha'nın oluşturduğu bu komplekse mikro işlemci kompleks (Mikroprocessor complex) adı verilir. Pre-miRNA molekülü bir nükleer taşıma reseptörü olan Exportin 5 ve nükleer bir protein olan RAN-GTP'ye bağımlı şekilde sitoplazmaya taşınır. Sonrasında, premiRNA'lar sitoplazmada RNAaz III enzim ailesinden Dicer adlı endonükleaz ile

kesilerek 18-24 nükleotid uzunluğunda çift zincirli miRNA (miRNA dubleksine) çevrilir. Dicer, aynı zamanda RNA ile tetiklenmiş susturma kompleksi (RNA-induced silencing complex; RISC) oluşumunu başlatır (şekil 2). MikroRNA'lar, RISC kompleksine entegre olduktan sonra, ya argonaute proteinleri yardımıyla mRNA'nın yıkımına ya da protein translasyonunun baskılanmasına neden olarak fonksiyon görürler (3,4).



Şekil 1. MikroRNA'ların biyogenezi (2)

MikroRNA'lar fonksiyonlarını kendi nükleotid dizilerine komplementer hedef genleri tanıma özelliği sayesinde gerçekleştirirler. Mikro- RNA'nın yapıya eklenmesi ile oluşan RISC kompleksi baz eşleşme özelliği ile mRNA'ya bağlanarak ilgili genin protein translasyonunun inhibisyonuna ve/veya mRNA'nın yıkılmasına sebep olur (Şekil 2). MikroRNA, hedef mRNA'nın 3'ucundaki translasyona uğramayan bölgesi (untranslated region-UTR) ya da hedef mRNA'nın ORF (open reading frame) bölgesine bağlanır. Bu bağlanma pozisyonu mikroRNA kompleksinin mRNA'ya nasıl komplementer olduğuna bağlıdır. 3'UTR bölgesine bağlanma kusurlu, tam olmayan, eksik komplementerliği ihtiva eder ve translasyonun baskılanması ile sonuçlanır. ORF bölgesi içine bağlanma ise kusursuz, tam komplementerliği gösterir ve Argonaute2 (Ago2) tarafından mRNA'nın yıkımı ile sonuçlanır. Eskiden fonksiyon görmediği düşünülen bu küçük moleküllerin günümüzde hücre diferansiyasyonu, proliferasyonu ve apopitoziste önemli kritik roller üstlendiği anlaşılmıştır (5). MikroRNA terimi ilk kullanıldığı günden beri normal ve malign hematopoezde mikro- RNA'ların rolünü anlamak için büyük çapta çalışmalar yapılmış ve sonucunda önemli gelişme kaydedilmiştir. Yapılan çalışmalar sonucunda bu küçük moleküllerin hematopoezde farklılaşma, çoğalma ve apoptoz (programlı hücre ölümü) gibi çok önemli hücresel olaylarda kritik öneme sahip olduğu gösterilmiştir (6).

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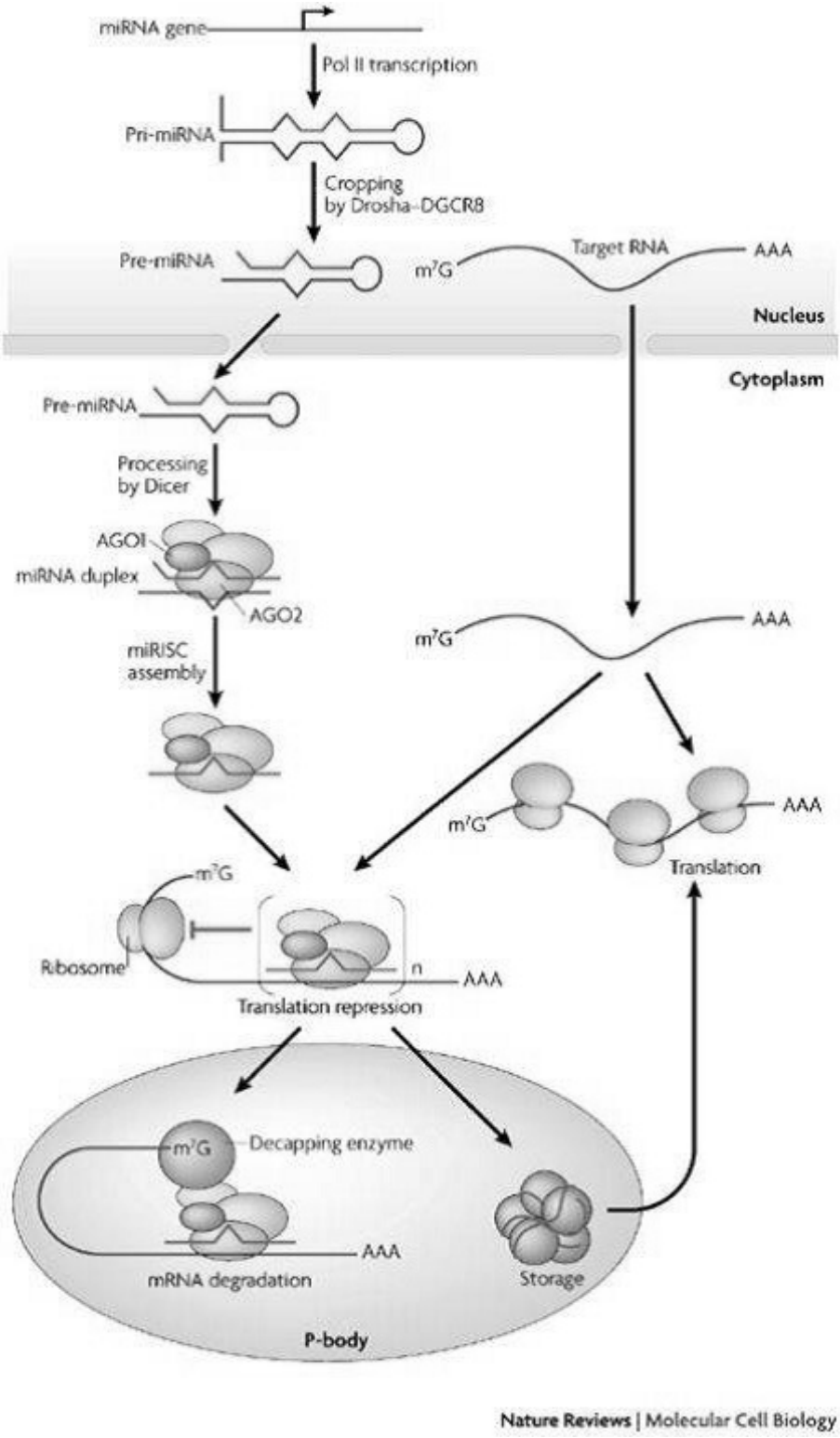
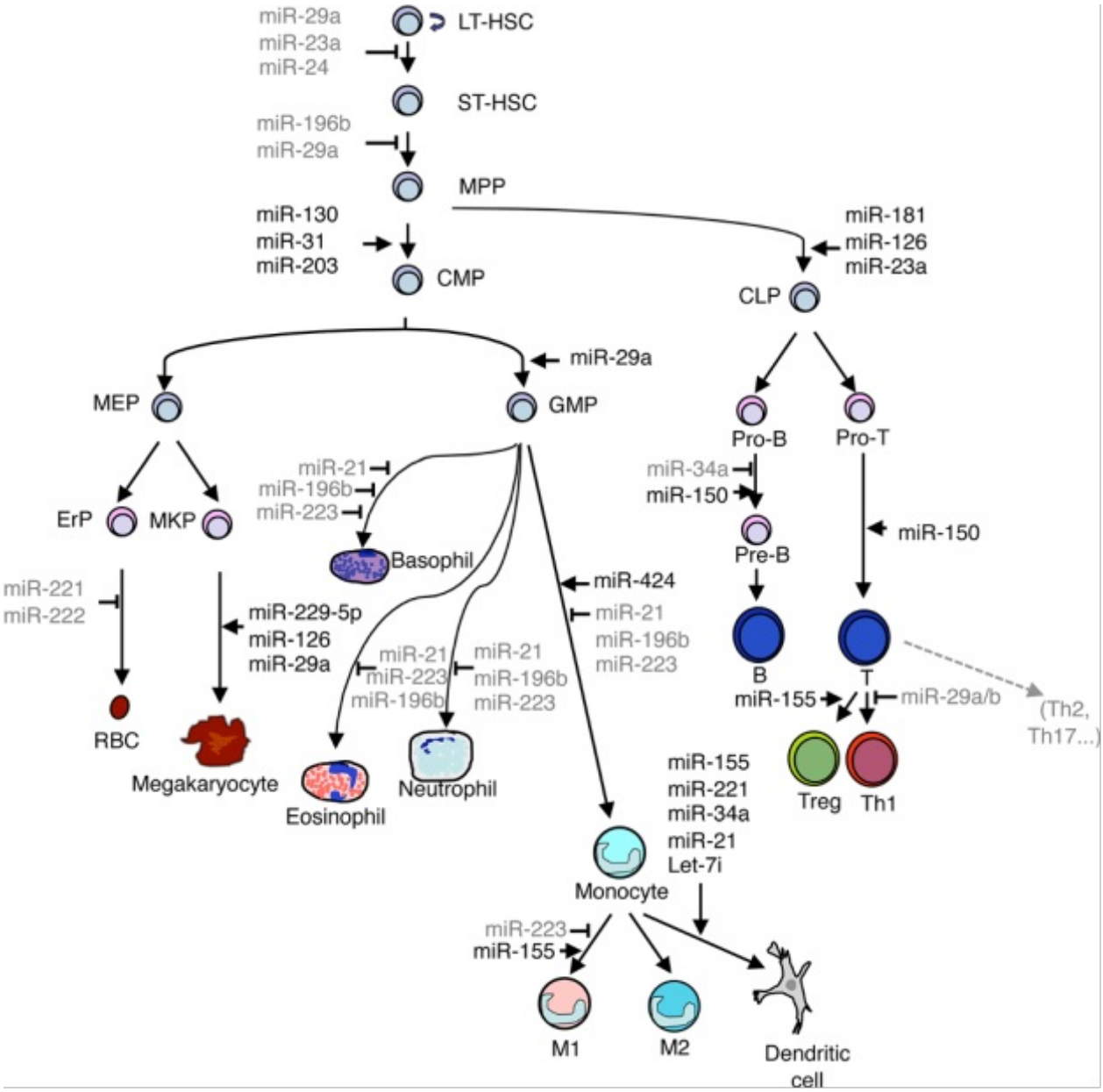


Şekil 2. RİSC kompleksi oluşumu (3)

Normal hemotopoezde mikroRNA'lar hemen her basamakta hematopoetik farklılaşmayı düzenleyerek son derece önemli rol oynarlar (Şekil 3).



Şekil 3. Hematopoezde görevli mikroRNA'ların ekspresyonu (1)

Normal ve maling hematopoezdeki mikroRNA ekspresyonları arasındaki ilişkiyi saptamak için giderek artan sayıda çalışma yapılmaktadır. Ancak eldeki verilerle şu anda hematolojik malignensilerde mikroRNA'nın direkt rolünü gösteren çok az sayıda veri vardır. Bunun birinci nedeni mikroRNA'ların çok sayıda farklı hedeflerinin olmasının yanı sıra mikroRNA'ların eşlik ettiği çok fazla sayıda karmaşık ve birbiri ile örtüşen yolların olmasıdır. Çalışmaların çoğunda hematolojik malignensilerde onkogenesize eşlik eden tek mikroRNA saptanma girişimi, çok sayıda ve anormal mikroRNA'ların varlığı nedeniyle karmaşık hale gelmektedir. mir-155 GC (germinal center) reaksiyonunu düzenler ve transgenik farelerde B hücreli malignensilere eşlik eder. Gerçekten de Diffuse large B-cell lymphoma (DLBCL), KLL, Hodgkin lenfoma ve Primer Mediasteneal B Hücreli lenfomada mir-155 artışı, bu mikroRNA'nın lenfomagenesiz de anahtar rol oynadığını göstermektedir. Ayrıca mir-155 miyelopoesizinde önemli bir düzenleyicisidir. Çünkü mir-155 myeloid ve eritroid farklılaşmayı bloke eder (Tablo 1). Transgenik farelerde mir-155'in anormal ekspresyonu miyeloproliferatif hastalık ile sonuçlanıp, eritroid/megakaryositik seride azalmaya neden olur. Sonuç olarak mir-155'in anormal ekspresyonu, çeşitli yollarda rol oynayarak hem myeloid hemde lenfoid malignensi oluşumuna eşlik eder. Mir-15a/16-1 tümor supresör ajan olarak kabul edilir. KLL vakalarının büyük çoğunluğunda azalır veya yok olur. Mir-181 sıçan B-lenfoid hücrelerinde fazlaca eksprese olur. Mir-181'in progenitor hücrelerde ektopik ekspresyonu B hücre oranında artışa neden olup hematopoetik serilerde farklılaşmayı inhibe ettiği düşünülmektedir. Gerçekten de mir-181 ailesi üyeleri AML ile yakın ilişkilidir. Çünkü mir-181 AML-M1, AML-M2 normal karyotiplerde artış gösterirken, yüksek riskli AML'de azalmışdır. Mir-181 ailesinin diğer üyesi mir-181b kötü prognozlu KLL de azalmıştır (7).

Tablo1. Normal hematopoezde mikroRNA'ların görevleri

MikroRNA	Fonksiyonları
Mir-150	B ve T hücrelerinin farklılaşmasının kontrolü Matur T ve B hücrelerinin ekspresyonu Öncül B hücrelerinin bloke edilmesi
Mir-155	B ve T hücrelerinin farklılaşmasının kontrolü T hücre aktivasyonu
Mir-221 ve Mir-222	Eritrosit farklılaşmasının bloke edilmesi
Mir-181a	B ve T hücrelerinin farklılaşmasının kontrolü Öncül hücrelerin inhibisyonu
Mir-223	Granülosit oluşununu artırmak Eritropoiesis oluşumunu artırmak
Mir-146	Lenfoidlerin farklılaşmasını bloke etmek
Mir-10a, Mir126,Mir-106,Mir-10b,Mir-17,Mir-20	Monosit farklılaşması ve olgunlaşması

Kanser progresyonu ve metaztazında Epitel- Mezenkimal Transition (EMT) önemli bir gelişimsel süreçtir. EMT'nin kendisi post transkripsiyonel modülatörler ve kompleks transkripsiyon ağları ile düzenlenir. MikroRNA'lar burada da son derece önemli kritik role sahiptir. Kanser oluşumunda kök hücre veya kök hücre özelliklerine sahip kanser hücrelerinin tümör oluşumu ve metastazından sorumlu olduğu düşünülmektedir. Ancak bu hücrelerin hangi mekanizmalarla kanser kök hücrelerine dönüştürüldüğü hangi hücresel mekanizma ve olaylarla düzenlendiği moleküler seviyelerde tam olarak anlaşılamamıştır. Bu süreçteki son gelişmeler kanser oluşumu ve progresyonu konusunda mikro-RNA'ların önemini ön plana çıkarmıştır (8).

miRNA'ların fonksiyonları indüklediği hücre tipiyle oldukça ilişkilidir, aynı miRNA farklı hücre tipinde ve stres durumlarında çok farklı fonksiyonlar gösterebilir. Fonksiyonları eksprese edilen mRNA'lara bağlıdır. Bu özel davranış şekilleri, özgül hastalıklarda miRNA'ların miRNA-tabanlı tedavilerde kullanılabilieceği fikrini de doğurmaktadır. Literatürde yer alan çalışmalarda bazı miRNA'ların anormal ekspresyonlarının myeloid malignansilerin patogenezinde rol oynadığı belirtilmektedir.

Bu çalışmada araştırılacak olan miRNA'ların ekspresyonları myeloid malignansilerin tedavisinde kullanılacak ilaç kaynaklarının saptanabilmesine, farklı myeloid malignansilerin gelişen hematopoezdeki farklı etkilerini, hastalık ilerlemesinde ve lösemik transformasyonlardaki rollerini göstererek araştırmaların hastalık patogenezini daha çok anlamamıza ve daha yeni miRNA-tabanlı tedavilerin gelişmesine yol açacağını, aynı zamanda da hastalık özgül miRNA'ların myeloprolifatif hastalıkların ayırıcı tanısında kullanılabileceğini düşünmekteyiz.

PROJEDEN BEKLENEN YAYGIN ETKİ

Hematolojik malignensi hastaları, antineoplastik tedaviler ve kanser komplikasyonlarının sonucu olarak yorgunluk, uykusuzluk, uyuşukluk, ağrı, nefes darlığı ve nöropati gibi fiziksel semptomlar gösterir. Sitopeni, enfeksiyonlar ve pıhtılaşma bozuklukları bu hastalarda yaygındır. Dahası, bu hastalar, yaşamının sonuna kadar yoğun antineoplastik ilaçlarla tedavi edilmektedir. Bu nedenlerden dolayı bu bireyler sürekli stress altındadır. Hematolojik malignensiler, yüksek morbidite ve mortaliteye yol açtığından erken tanı ve etkili tedavi oldukça önemlidir. Hastalığın erken tanı ve tedavisi, ülke ekonomisi, toplum sağlığı ve hasta bireylerin yaşam kalitesinin arttırılması açısından faydalı olabilir.

Kanserin gelişim sürecindeki moleküler genetik mekanizmalar çoğunlıkla her kanser tipi için farklılık göstermekte ve hala araştırılmaya devam etmektedir. Son yıllarda ise miRNA-kanser ilişkisi konusunda bir çok araştırma yayınlanmaktadır. Bu çalışmadan elde edilecek veriler önemli bir bilimsel katkı sağlayacaktır.

PROJEDE ÖNGÖRÜLEN/BEKLENEN BİLİMSEL/AKADEMİK ÇIKTI, SONUÇ VE ETKİLER

No	Çıktı/Sonuç/Etkiler
1	Bu proje sonucunda, çalışmamızın poster/sözlü bildiri yapılarak kongrede sunulması ve daha sonra SCI/SCI-E indeksli bir dergide araştırma makalesi olarak yayınlanması planlanmaktadır.

PROJEDE ÖNGÖRÜLEN/BEKLENEN EKONOMİK/TİCARİ/SOSYAL ÇIKTI, SONUÇ VE ETKİLER

No	Çıktı/Sonuç/Etkiler
1	Projemiz sayesinde Myeloid Hematolojik Malignansilerde mikroRNA ekspresyon seviyelerinin belirlenmesi ile yeni tedavi yöntemlerinin geliştirilmesine olanak sağlanacaktır.

PROJEDE ÖNGÖRÜLEN/BEKLENEN ARAŞTIRMACI YETİŞTİRİLMESİ VE YENİ PROJE(LER) OLUŞTURMA ÇIKTI, SONUÇ VE ETKİLER

No	Çıktı/Sonuç/Etkiler

No	Çıktı/Sonuç/Etkiler
	Bu proje yüksek lisans öğrencimize moleküler tekniklerin (RNA eldesi, cDNA sentezi, PCR vb.) tıpta kullanımı konusunda teorik bilgi ve pratik beceri kazandıracaktır. Önemli bir sağlık sorunu olan myeloid malignansilerde , mikroRNA ekspresyon çalışması ile kanserin oluşum sürecinde yer alan hücresel olaylardaki değişimleri ve bunların etkilerini kavramayı sağlayacaktır.
1	Edinilen bilgiler doğrultusunda Myeloid Hematolojik Malignansi karsinogenezine yönelik yeni moleküler yaklaşımlar ve yeni yöntemler geliştirilmesi söz konusu olabilecektir. Hastalara ait kemik iliği örneklerinden elde edilecek Hastalardan elde edilecek RNA/cDNA ların çalışma haricinde kalan kısımları bankalanarak birçok başka araştırmaya konu olmaya devam edecektir.

YÖNTEM

Bu araştırmada myeloid malignansi hastalarında, hematopoezde görevli genlerin ekspresyonunu düzenleyen, miR-155, miR181a, miR-221, miR-222, miR-223 ve miR-451'in ekspresyonları, reel-time PCR yöntemi kullanılarak araştırılacaktır. Çalışmaya ait iş planı ve yöntem dizgesi aşağıda verilmiştir:

MEÜ Tıp Fakültesi İç Hastalıkları A.D. Hematoloji Bilim Dalı tarafından gerçekleştirilecek işlemler:

- Hematoloji Bilim Dalı'na başvuran ve Myeloid Malignansi tanısı alan hastalardan (bilgilendirilmiş onam formu ile hasta rızası alındıktan sonra) kemik iliği aspirasyon örneklerinin toplanması(n=50).

MEÜ Tıp Fakültesi Tıbbi Biyoloji Anabilim Dalı'nda gerçekleştirilecek işlemler:

- Kemik iliği aspiratlarından Total RNA eldesi (RNaesy/Trizol yöntemi)
- cDNA eldesine uygun RNA'ların dansitometrik yöntemle belirlenmesi
- Uygun miktar ve saflıktaki RNA'lardan cDNA sentezi
- Sentezlenen cDNA'lardan reel time PCR ile miR-155, miR181a, miR-221, miR-222, miR-223 ve miR-451 ekspresyonlarının saptanması
- Elde edilen ekspresyon verilerinin "Comparative CT (ΔΔCT)" yöntemi ile analizi
- Saptanan ekspresyon verilerinin bioistatistiksel yöntemler ile analizi (MEÜ Tıp Fakültesi Bioistatistik ve Tıbbi Bilişim Anabilim Dalı danışmanlığında)

1-Çalışma Grubunun Oluşturulması:

Çalışma grubumuza, 2017-2018 yılları arasında Mersin Üniversitesi Tıp Fakültesi Araştırma ve Uygulama Hastanesi İç Hastalıkları Bölümü Hematoloji Bilim Dalı'nda myeloid malignansi tanısı almış ya da ön tanısı ile gelmiş 50 hasta dahil edilecektir. Endojen kontrol grubu olarak hsa-miR-26b-5p'nin ekspresyonu kullanılacaktır. Hastalara ait kemik iliği örneklerinden ilgili genetik analizler Mersin Üniversitesi Tıp Fakültesi Tıbbi Biyoloji Anabilim Dalı Moleküler Genetik Tanı Laboratuvarlarında gerçekleştirilecektir. Tez çalışmamız içerisinde yer alan moleküler çalışmalarda Real Time PCR (ABI 7500, Applied Biosystem) cihazı ve SDS 2.0.6 software (Aapplied Biosysteems) kullanılacaktır. Hasta grubuna ait bireylere araştırmayı kabul ettiğine dair bilgilendirilmiş onam formu imzalatılacaktır.

2- Total RNA İzolasyonu:

Myeloid malignansi tanısı almış veya tedavi süreci devam eden 50 hastadan EDTA'lı tüplere alınan 3-4 ml kemik iliği örnekleri RNeasy Kit (Qiagen Inc., Valencia, CA) yada TRIZOL (Invitrogen) kullanılarak manuel yöntemle total RNA izolasyonu gerçekleştirilecektir. RNA elde edilene kadar örnekler -80°C'de muhafaza edilecektir.

3- cDNA Sentezi:

Her bir RNA örneğinden cDNA sentezi yapılacaktır. cDNA sentezi öncesinde tüm RNA örneklerinin miktar tayinleri (O.D.280nm'de spektrofotometrik yöntemle) yapılarak saflık oranları belirlenecektir. Miktar ve saflık oranları cDNA eldesine uygun olan RNA'lardan cDNA sentezi standart protokole göre yapılacaktır.

4.Real-time kantitatif PCR ile gen ekspresyonlarının belirlenmesi:

Sentez edilen cDNA'lardan hematopoetik miRNA'lar olarak literatürde tanımlanan;

- miR-155
- miR181a
- miR-221
- miR-222
- miR-223
- miR-451

ekspresyon düzeylerinin rölatif kantitasyonu her bir miRNA için ayrı ayrı belirlenen özgül primer ve prob dizileri kullanılarak Real Time PCR (ABI 7500, Applied Biosystem) ile gerçekleştirilecektir. Her miRNA'ya özgü primer ve problar literatürden yararlanılarak sentez edilecektir. Ekspresyon düzeylerinin kantitasyonunu ise "Comparative CT (ΔΔCT)" analizi ile yapılacaktır. Yöntem; hedef gen ve endojen kontrol hsa-miR-26b-5p ile pozitif RNA örneğine (TaqMan Human Control Total RNA-4307281 den sentezlenen cDNA) göre ekspresyon düzeylerindeki farklılıkları belirleme şeklinde çalışmaktadır. Endojen kontroller, PCR karışımına yüklenen cDNA örneklerinde ekspresyon miktarlarındaki farklılıkları hedef genlerin ekspresyon düzeyleri ile normalize etmek için kullanılmaktadır. Reaksiyon sonrası elde edilen ΔΔCT değerleri kullanılarak $2^{-\Delta\Delta CT}$ hesaplanacak ve endojen kontrol ile referans RNA ya karşı araştırılan genin ekspresyon düzeyleri belirlenecektir.

Elde edilecek veriler istatistiksel yöntemler kullanılarak test edilecektir.

YÖNETİM DÜZENİ(İŞ PAKETLERİ(İP), GÖREV DAĞILIMI VE SÜRELERİ)

No	İş Tanımı	Uygulayıcılar	Aylar																							
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	Malzemelerin İstemi ve Temini	CEMİLE ERSÖZ	✔	✔	✔	✔	✔	✔	✔	✔																
2	Hasta bireylerden kemik iliği örneklerinin temini	MUSTAFA ERTAN AY CEMİLE ERSÖZ	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔												
3	Kemik iliği örneklerinden RNA eldesi ve cDNA sentezi	CEMİLE ERSÖZ						✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔								
4	Real-Time PCR ile miRNA ekspresyon düzeylerinin incelenmesi	CEMİLE ERSÖZ						✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔								
5	Sonuçların değerlendirilmesi ve yorumlanması	MUSTAFA ERTAN AY CEMİLE ERSÖZ																	✔	✔	✔	✔				

No	İş Tanımı	Uygulayıcılar	Aylar																					
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
6	Yüksek lisans tez yazım aşaması	CEMİLE ERSÖZ																	✔	✔	✔	✔	✔	✔	✔	✔

BAŞARI ÖLÇÜTLERİ VE RİSK YÖNETİMİ

1. İş Paketi

İş Tanımı:	Malzemelerin İstemi ve Temini	**Proje Başarısındaki Önemi:**	25

Hedef:

Tez çalışmasının sorunsuz ve en iyi şekilde yürütülebilmesi için malzeme istemi ve temininin çalışmaya başlanmadan önce tedarik edilmesi.

Başarı Ölçütü:

%95

2. İş Paketi

İş Tanımı:	Hasta bireylerden kemik iliği örneklerinin temini	**Proje Başarısındaki Önemi:**	25

Hedef:

Çalışmada araştırılacak özelliklerin doğru bir şekilde analizinin yapılabilmesi için çalışma prosedürüne uygun miktarda kemik iliği örneklerinin temin edilmesi gerekmektedir.

Başarı Ölçütü:

%95

3. İş Paketi

İş Tanımı:	Kemik iliği örneklerinden RNA eldesi ve cDNA sentezi	**Proje Başarısındaki Önemi:**	15

Hedef:

Tüm Kİ örneklerinden uygun miktar ve saflıkta, PCR için uygun RNA eldesi ve bunlardan cDNA sentezi

Başarı Ölçütü:

%95

4. İş Paketi

İş Tanımı:	Real-Time PCR ile miRNA ekspresyon düzeylerinin incelenmesi	**Proje Başarısındaki Önemi:**	15

Hedef:

RT-PCR ile myeloid malignansilerde hemetopoetik miRNA ekspresyon düzeyleri incelenecektir.

Başarı Ölçütü:			
%95			
5. İş Paketi			
İş Tanımı:	Sonuçların değerlendirilmesi ve yorumlanması	**Proje Başarısındaki Önemi:**	10
Hedef:			
miRNA ekspresyon düzeyleri incelenerek hematolojik malignansiler hakkında bilgi sahibi olmak.			
Başarı Ölçütü:			
%95			
6. İş Paketi			
İş Tanımı:	Yüksek lisans tez yazım aşaması	**Proje Başarısındaki Önemi:**	10
Hedef:			
MEÜ Sağlık Bilimleri Enstitüsü Lisansüstü Eğitim Yönetmeliğinde belirtilen "tez yazım kurallarına" uygun olarak proje çıktılarının yüksek lisans tezi olarak yazılması.			
Başarı Ölçütü:			
%100			

RİSK YÖNETİMİ TABLOSU VE TEDBİRLER

1. Risk ve Tedbir	
İş Tanımı:	Malzemelerin İstemi ve Temini
Risk	
Beklenen süre içerisinde gerekli malzeme temininde gecikme yaşanabilir yada gelen malzemeler bozuk yada kırılmış olabilir.	
B Planı	
Daha önce benzer çalışmalarda kullanılan malzemelerden kullanılacaktır.	
2. Risk ve Tedbir	
İş Tanımı:	Hasta bireylerden kemik iliği örneklerinin temini
Risk	
Yeterli miktarda kemik iliği örneği alınamayabilir yada kliniğe istenilen sayıda hasta gelmemiş olabilir.	
B Planı	

Çalışmaya öncelikle yeni tanı almış hastaların dahil edilmesi düşünülmekle birlikte, gerekli görüldüğü koşullarda (hasta sayısının yetersizliği vb..) takip hastalarının da çalışmaya dahil edilmesi planlanmaktadır.

3. Risk ve Tedbir

İş Tanımı:	Kemik iliği örneklerinden RNA eldesi ve cDNA sentezi

Risk

Örneklerin transportu yada saklanmasındaki aksaklıklardan kaynaklı kemik iliği örneklerinden ekspresyon analizine uygun miktar ve kalitede RNA eldesi mümkün olmayabilir.

B Planı

Farklı izolasyon ve cDNA sentez protokolleri denenecek yada yeni hasta örneği temin edilmeye çalışılacaktır.

4. Risk ve Tedbir

İş Tanımı:	Real-Time PCR ile miRNA ekspresyon düzeylerinin incelenmesi

Risk

1-RT-PCR cihazı herhangi bir nedenden dolayı bozulabilir.

2-Çalışmaya dahil edilen miRNA ların hücre spesifik ekspresyonlarının çok düşük düzeyde olmasından dolayı değerlendirme yapılamayabilir.

B Planı

1-Real-Time cihazında meydana gelecek herhangi bir aksaklık sonucu çalışmamızın geri kalan kısmında MEİTAM'daki Real-Time cihazı kullanılarak deneyler tamamlanacaktır.

2-Çalışmaya, proje bütçesi imkanları doğrultusunda yeni primer/problar temin edlmesi mümkün olursa diğer hematopoetik miRNA'lar dahil edilecektir.

5. Risk ve Tedbir

İş Tanımı:	Sonuçların değerlendirilmesi ve yorumlanması

Risk

Sonuçların değerlendirilmesi ve yorumlanması aşamasında istatiksel veriler ve sonuçlar arasında uyumsuzluk görülebilir.

B Planı

Yeni oluşturulacak istatistiki veriler doğrultusunda hasta sayısı artırılarak yada azaltılarak çalışa devam ettirilecektir.

6. Risk ve Tedbir

İş Tanımı:	Yüksek lisans tez yazım aşaması

Risk

Tez yazım aşaması sırasında herhangi bir donanım aksaklığından dolayı elde edilen bilgiler silinebilir.
B Planı
Tez defterine kaydedilen bilgiler doğrultusunda tez yazımına devam edilecektir.

PROJE ÇIKTILARININ PAYLAŞIMI VE YAYILIMI

#	Faaliyet Türü	Paydaş / Potansiyel Kullanıcılar	Tarih	Saat	Yer
1	SCI veya SCI Expanded Dergilerinde Yayımlanmak Üzere Makale	M.Ertan AY, Cemile ERSÖZ, Anıl Tombak, M.Emin Erdal, Özlem İzci Ay	13-03-2018	13:25	SCI veya SCI Expanded

ARAŞTIRMA OLANAKLARI

#	Mevcut Altyapı / Ekipman Türü, Modeli (Laboratuvar, Araç, Makine-Teçhizat vb.)	Mevcut Olduğu Kurum / Kuruluş	Projede Kullanım Amacı
1	Tıbbi Biyoloji Laboratuvarı	Mersin Üniversitesi	Deneysel çalışmaların yürütüldüğü bölüm
2	ABI Prism 7500 Real-Time PCR System (Applied Biosystems) cihazı	Mersin Üniversitesi	miRNA ekspresyon analizi
3	PCR	Mersin Üniversitesi	cDNA eldesi
4	Mikrosantrifüj (Hermle, Z160M)	Mersin Üniversitesi	Eppendorf tüplerinin santrifügasyon işlemi
5	Vorteks (VELP)	Mersin Üniversitesi	Maddelerin homojen olarak karıştırılması
6	Mikropipet Seti (Eppendorf)	Mersin Üniversitesi	Örneklerin istenilen miktarlarda alınması

PROJE YÜRÜTÜCÜSÜNÜN TÜBİTAK DESTEKLİ PROJELERİ

1. Sitokinlerle Aktive Edilmiş İnsan Astroglial Kültürlerinde Eritropoetinin Nörotrofik Faktörler ve İndüklenebilir-Nitrik Oksid Sentaz (iNOS) Üretimleri Üzerine Etkisi, TÜBİTAK, Proje No: 4, 0 TL, Araştırmacı, **Devam ediyor**.
2. Erişkin Olfaktör Nörogenezi'nde DNA Metilasyon ve Demetilasyon Dinamiklerinin Olası Rolü, TÜBİTAK, Proje No: 8, 0 TL, Araştırmacı, **Devam ediyor**.

PROJE YÜRÜTÜCÜSÜNÜN DİĞER PROJELERİ (DPT, BAP, FP6-7 VB.)

1. İzmir İli ve Çevresindeki Hastanelerde Doğum Yapan 35 yaş ve Üzeri Kadınların. Down Sendromlu Çocuk Doğurma Sıklığının Belirlenmesi , BAP, Proje No: 1, 0 TL, Araştırmacı, **Devam ediyor**.
2. Down Sendromunun İn-Situ Hibridizasyon Yöntemi ile Tanısı. , BAP, Proje No: 2, 0 TL, Yürütücü, **Devam ediyor**.
3. Gastrik Kanser Patogenezinde p21, p27, p57 Siklin Bağımlı Kinaz İnhibitör Genleri İle p53 ve p73 Tümör Supressör Genlerinin Moleküler Düzeyde Araştırılması, BAP, Proje No: 3, 0 TL, Araştırmacı, **Devam ediyor**.
4. Tekrarlayan gebelik kayıplarında Death Receptor-4 (DR-4, TRAIL-R1) gen polimorfizmlerinin araştırılması., BAP, Proje No: 5, 0 TL, Yürütücü, **Devam ediyor**.
5. Down Sendromu Öyküsü Olan Annelerde Folat/Homosistein Metabolizmasında Görevli Gen Polimorfizmlerinin Araştırılması, BAP, Proje No: 6, 0 TL, Araştırmacı, **Devam ediyor**.
6. İrritabl barsak sendromlu hastalarda leptin ve leptin reseptör gen polimorfizmlerinin araştırılması, BAP, Proje No:

7, 0 TL, Yürütücü, **Devam ediyor**.
7. Miyeloid Hematolojik Malignensilerde miRNA Oluşum Yolağındaki Genlerin Ekspresyon Düzeylerinin Araştırılması, BAP, Proje No: 2015-TP3-1205, 15000 TL, Yürütücü, **Devam ediyor**.

PROJE EKİBİNİN ÖNERİLEN PROJE İLE İLGİLİ PROJELERİ

1. Gastrik Kanser Patogenezinde p21, p27, p57 Siklin Bağımlı Kinaz İnhibitör Genleri İle p53 ve p73 Tümör Supressör Genlerinin Moleküler Düzeyde Araştırılması, BAP, Proje No: 3, 0 TL, Araştırmacı, **Devam ediyor**.
2. Tekrarlayan gebelik kayıplarında Death Receptor-4 (DR-4, TRAIL-R1) gen polimorfizmlerinin araştırılması., BAP, Proje No: 5, 0 TL, Yürütücü, **Devam ediyor**.
3. İrritabl barsak sendromlu hastalarda leptin ve leptin reseptör gen polimorfizmlerinin araştırılması, BAP, Proje No: 7, 0 TL, Yürütücü, **Devam ediyor**.
4. Erişkin Olfaktör Nörogenezi'nde DNA Metilasyon ve Demetilasyon Dinamiklerinin Olası Rolü, TÜBİTAK, Proje No: 8, 0 TL, Araştırmacı, **Devam ediyor**.

PROJE YÜRÜTÜCÜSÜNÜN SON 5 YILDA YAPTIĞI YAYINLAR

1. Camkurt, M.; Karababa, F.; Erdal, M.; Bayazit, H.; Kandemir, S.; Ay, M.; Kandemir, H.; Ay, O.; Cicek, E. Investigation of Dysregulation of Several MicroRNAs in Peripheral Blood of Schizophrenia Patients. *CLINICAL PSYCHOPHARMACOLOGY AND NEUROSCIENCE*, **2016**, *14*, 256-260.
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BÜTÇE PLANI

Proje Süresi	24 AY
Proje Bütçesi	11.000,00

Ay	Makine Teçhizat (%100)	Sarf Malzeme (%100)	Yolluk ve Konaklama Giderleri (%50)	SGK'lı Personel Çalıştırma (%0)	Hizmet Alımları (%50)	Toplam (%0)
3	0 TL	6.000 TL	0 TL	0 TL	0 TL	6.000 TL
6	0 TL	5.000 TL	0 TL	0 TL	0 TL	5.000 TL
9	0 TL	0 TL	0 TL	0 TL	0 TL	0 TL
12	0 TL	0 TL	0 TL	0 TL	0 TL	0 TL
15	0 TL	0 TL	0 TL	0 TL	0 TL	0 TL
18	0 TL	0 TL	0 TL	0 TL	0 TL	0 TL
21	0 TL	0 TL	0 TL	0 TL	0 TL	0 TL
24	0 TL	0 TL	0 TL	0 TL	0 TL	0 TL
Toplam	0 TL	11.000 TL	0 TL	0 TL	0 TL	11.000 TL

HARCAMA GEREKÇELERI

Makine Teçhizat	YOK
Sarf Malzeme	1.GeneMATRIX UNIVERSAL RNA Purification Kit (Kit for purification of total RNA) E3598-02 2.OneStep RT-PCR kit E0803-02 3.0.2 umol (200 nmol) Primer MOPC (Yield 8) 4000-7000 Test MCRGN-003 4.5x HOT FIREPol® EvaGreen® qPCR Supermix UNG 08-36-00001 5.0.1ml qPCR 96 well plate (ABI- Type) NB-Q96-LP 6.20mM of each dNTP Mix (1,25 ml) 02-31-00100 7.TRIS HCL, Ultra Pure TRS002 8.M-MLV Reverse Transcriptase 06-21-010000 9.Ribonuclease Inhibitor E4210-01 10.DIETHYLPYROCARBONATE (DEPC) DEP001.R 11.Certified 0.5ml Thin Wall Tubes & Cap (Flat) Natural I1405-8100 12.1.5ml TubeOne® Mikrosantrifüj Tüpü (Naturel Eppendorf Tüp) S1615-5500 13.10 ul Graduated Tip (Beyaz Uç) 2.5 ul, 10 ul (Katalog ürünüdür) S1111-3000 14.200 µl UltraPoint (Sarı Uç) Graduated Tip 10 µl,50 µl,100 µl (Katalog Ürünüdür) S1113-1006 15.1000µl Graduated (Mavi Uç) (Katalog ürünüdür) S1111-6001
Yolluk ve Konaklama Giderleri	YOK
Hizmet Alımları	YOK

PROJE KAPATMA KOŞULLARI

Fen, Mühendislik, Sağlık Bilimleri, Sosyal, Beşeri ve İdari Bilimler

Projeden elde edilen veriler ile hazırlanmış, Science Citation Index (SCI), SCI-Expanded, Arts and Humanities Citations Index (AHCI), Social Science Citation Index (SSCI), Matematik, Sosyal, Beşeri ve İdari Bilimlerde uluslararası alan endeksi kapsamında bulunan dergilerde **en az bir adet** yayın yapma veya ulusal/uluslararası kongrelerde **en az bir adet** sözlü/poster sunum yapma zorunluluğu esastır.

Eğitim Bilimleri ve Spor Bilimleri

Projeden elde edilen veriler ile hazırlanmış, Science Citation Index (SCI), SCI-Expanded, Arts and Humanities Citations Index (AHCI), Social Science Citation Index (SSCI), Australian Education Index, British Educational Journals Indexed in Erich, Education Full Text Database Coverage List kapsamında bulunan dergilerde **en az bir adet** yayın yapma veya ulusal/uluslararası kongrelerde **en az bir adet** sözlü/poster sunum yapma zorunluluğu esastır.

Mimarlık

Projeden elde edilen veriler ile hazırlanmış, Science Citation Index (SCI), SCI-Expanded, Arts and Humanities Citations Index (AHCI), Social Science Citation Index (SSCI), Architectural Publications Index, Architectural Periodicals Index, Art Index, Design and Applied Art Index, Avery Index to Architectural Periodicals, Ergonomics Abstracts, Earthquake Engeenering Abstracts kapsamında bulunan dergilerde en **az bir adet** yayın yapma veya ulusal/uluslararası kongrelerde **en az bir adet** sözlü/poster sunum yapma zorunluluğu esastır.

Güzel Sanatlar

Projeden elde edilen veriler ile hazırlanmış, sanat kurullu/hakemli/küratörlü olmak üzere, ulusal/uluslararası müze, tiyatro, tanınmış sanat kurumları, tanınmış sanat galerileri veya festivallerde, en az bir adet kişisel/karma ortak etkinlik (sergi, bienal, gösteri, dinleti, gösterim) veya sanat kurullu/hakemli/küratörlü olmak üzere festival, çalıştay, bienal gibi etkinliklere **en az bir adet** eserle katılmak veya ulusal/uluslararası kongrelerde en az bir adet sözlü veya poster sunum yapma veya ulusal/uluslararası hakemli dergilerde **en az bir adet** yayın yapma zorunluluğu esastır. Sanat ve tasarım alanlarında üretilen eserler birden fazla kopyaları üretilmiş ve farklı yerlerde sergilenmiş olsalar da sadece bir kez değerlendirmeye tabi olurlar.

Yukarıda belirtilen koşulları okudum ve kabul ediyorum

Başvuru Tarihi	04.11.2016

PROJEDE GÖREV ALANLAR

T.C. Kimlik No	Adı Soyadı	Birimi	Görevi	İmza
22393888362	MUSTAFA ERTAN AY	TIP FAKÜLTESİ	Proje Yürütücüsü	
24946560084	CEMİLE ERSÖZ	TIP FAKÜLTESİ	Yüksek Lisans Öğrencisi	

BIRIM AMIRI

Prof. Dr. Ahmet Hakan ÖZTÜRK

İmza

BAŞVURU FORMU EKLERI

Ek 1. 2017-1-TP2-2037 faturalar
Ek 2. 2017-1-TP2-2037 destek materyaller
Ek 3. 2017-1-TP2-2037 kurum kurul izin
Ek 4. 2017-1-TP2-2037 etik kurul raporu
Ek 5. CV 2017-1-TP2-2037 MUSTAFA ERTAN AY
Ek 6. CV 2017-1-TP2-2037 CEMİLE ERSÖZ

BM Laboratuvar Sistemleri

MERSİN ÜNİVERSİTESİ
TIP FAKÜLTESİ - TIBBİ BİYOLOJİ AD
PROJE NO:
SAYIN: DR. CEMİLE ERSÖZ

Tarih: 28/10/16

Firmamızdan talep etmiş olduğunuz ürünlere ait teklifimizi aşağıda bilgilerinize sunarız.

PROFORMA FATURA

No	Ürün	Marka	Ölçü	KDV	Miktar	Fiyat	Son Fiyat
1	GeneMATRIX UNIVERSAL RNA Purification Kit (Kit for purification of total RNA) E3598-02	EURx	100 Preps	8	1	1.040,00TL	1.040,00TL
2	OneStep RT-PCR kit E0803-02	EURx	100 Rxn.	8	1	1.430,00TL	1.430,00TL
3	0.2 umol (200 nmol) Primer MOPC (Yield 8) 4000-7000 Test MCRGN-003	Macrogen	Baz	18	500	3,35TL	1.675,00TL
4	5x HOT FIREPol® EvaGreen® qPCR Supermix UNG 08-36-00001	Solis	250 Rxn	8	4	388,00TL	1.552,00TL
5	0.1ml qPCR 96 well plate (ABI- Type) NB-Q96-LP	New Bioscience	10 Ad/pk	18	5	115,00TL	575,00TL
6	20mM of each dNTP Mix (1,25 ml) 02-31-00100	Solis	100 umol	8	1	296,00TL	296,00TL
7	TRIS HCL, Ultra Pure TRS002	Bioshop	500 gr	18	1	330,00TL	330,00TL
8	M-MLV Reverse Transcriptase 06-21-010000	Solis	10000 U	8	2	650,00TL	1.300,00TL
9	Ribonuclease Inhibitor E4210-01	EURx	7 500 U	8	2	585,00TL	1.170,00TL
10	DIETHYLPYROCARBONATE (DEPC) DEP001.R	Bioshop	100 ml	18	1	1.126,78TL	1.126,78TL
11	Certified 0.5ml Thin Wall Tubes & Cap (Flat) Natural I1405-8100	Starlab	1000 Ad/pk	18	1	136,00TL	136,00TL
12	1.5ml TubeOne® Mikrosantrifüj Tüpü (Naturel Eppendorf Tüp) S1615-5500	Starlab	500 Ad/pk	18	1	62,00TL	62,00TL
13	10 ul Graduated Tip (Beyaz Uç) 2.5 ul, 10 ul (Katalog ürünüdür) S1111-3000	Starlab	1000 Ad/pk	18	1	100,00TL	100,00TL
14	200 µl UltraPoint (Sarı Uç) Graduated Tip 10 µl,50 µl,100 µl (Katalog Ürünüdür) S1113-1006	Starlab	1000 Ad/pk	18	1	78,00TL	78,00TL
15	1000µl Graduated (Mavi Uç) (Katalog ürünüdür) S1111-6001	Starlab	1000 Ad/pk	18	1	95,00TL	129,22TL
							11.000,00TL

Saygılarımızla

Yalnız KDV Dahil Onbirbin TL'dir.
Fiyatlarımız 28/11/2016 tarihine kadar geçerlidir.

BM YAZILIM DANIŞ. VERİ İLET.
LAB. SİS. EĞT.SAN.VE TİC. LTD.ŞTİ.
Reşit Galip Cad. No: 74/7 06700 Çankaya/ANK
Tel: 0312 447 22 79-80 Fax: 0312 447 92 07
V.D. Kavaklıdere: 178 0565 067

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Journal of Hematology & Oncology

RESEARCH Open Access

MiR-124-3p/B4GALT1 axis plays an important role in SOCS3-regulated growth and chemo-sensitivity of CML

Yu-xiao Liu[1†], Li Wang[2,3†], Wen-jia Liu[4†], Hai-tao Zhang[1], Jing-hui Xue[1*], Zhi-wen Zhang[1] and Chun-ji Gao[3*]

Abstract

Background: Abnormal expression of SOCS3 has been implicated in myeloproliferative neoplasms, but the role of SOCS3 in the pathogenesis of leukemia remains largely unknown. Here, we examined the function of SOCS3 in the growth and chemo-sensitivity of chronic myeloid leukemia (CML) and explored the involved mechanisms.

Methods: Expression levels of SOCS3 in several leukemia cell lines and bone marrow mononuclear cells (BMNCs) from CML patients were determined using quantitative real-time PCR (qPCR) and Western blotting (WB). The roles of SOCS3 in the proliferation, apoptosis, and drug resistance of CML cells were examined by clonogenic progenitor cell assay, flow cytometry, and CCK-8 assay. A detailed analysis of the underlying mechanism of SOCS3 in K562 cells was performed using the Human HT-12 v4 Expression BeadChip, which has more than 48000 gene probes including 600 microRNAs (miRNA) probes. The correlation between the mRNA expression of SOCS3 and miR-124-3p in BMNCs from 30 CML patients was tested by qPCR and analyzed by Pearson correlation and linear regression analysis. The potential target of miR-124-3p in CML cells was explored using the luciferase reporter assay, qPCR, and WB. The effect of SOCS3 on the miR-124-3p/B4GALT1 axis was investigated by qPCR, WB, CCK-8 assay, and tumorigenicity assays in nude mice.

Results: SOCS3 was down-regulated in CML cell lines and most of BMNCs from CML patients, and the expression level of SOCS3 was associated with the inhibition of cell proliferation and drug resistance of CML cells. Over-expression of SOCS3 in K562 cells inhibited the expression of leukemia-specific genes and promoted the expression of some miRNAs, among which miR-124-3p was the highest. SOCS3 over-expression enhanced the expression of miR-124-3p and vice versa. The mRNA expression of miR-124-3p and SOCS3 in BMNCs from 30 CML patients was positively correlated. Consistently, the tumor suppressing effects of SOCS3 were partially neutralized by the miR-124-3p inhibitor. B4GALT1 was downstream of miR-124-3p and regulated by SOCS3/miR-124-3p in vitro. Furthermore, SOCS3 over-expression could inhibit the growth and B4GALT expression of K562 cells in vivo.

Conclusions: SOCS3/miR-124-3p/B4GALT1 axis plays an important role in the pathogenesis of CML.

Keywords: SOCS3, miR-124, B4GALT1, Leukemogenesis, Chemo-sensitivity

* Correspondence: xuejh629@163.com; gaochunji@hotmail.com
†Equal contributors
[1]Department of Neurosurgery, The First Affiliated Hospital of Chinese PLA General Hospital, 51 Fushi Road, Beijing 100048, People's Republic of China
[3]Department of Hematology, Chinese PLA General Hospital, 28 Fuxing Road, Beijing 100853, People's Republic of China
Full list of author information is available at the end of the article

Background

Suppressor of cytokine signaling (SOCS) is a protein family of eight members (SOCS1–7 and CIS) that form a classical negative feedback system to regulate cytokine signal transduction [1]. By regulating the cytokine-driven STAT activation pathway, SOCS3 likely has an important role in development, allergic responses, and tumorigenesis [2–4]. Methylation of the SOCS3 promoter and reduced gene expression of SOCS3 have been documented in numerous tumors such as breast cancer, lung cancer, and liver cancer [5–7]. SOCS3 is highly conserved among vertebrates and has been considered to be a transcriptional regulator in the hematopoietic system. SOCS3$^{-/-}$ mice die in utero because of fetal liver erythrocytosis, and over-expression of SOCS3 blocks fetal liver erythrocytosis, implying that SOCS3 plays a critical role in the negative regulation of hematopoiesis [8, 9].

Evidence suggests a potential role of SOCS3 in the pathogenesis of leukemia. For example, Capello et al revealed that inactivation of SOCS3 was frequent in Ph-negative chronic myeloproliferative disorders (CMPD) [10]. Al-Jamal et al. reported that down-regulation of SOCS3 was involved in the resistance of CML cells to imatinib [11]. However, the exact function of SOCS3 in hematological malignancies remains unclear. A better understanding of the function and underlying molecular mechanisms of SOCS3 will contribute to the precision medicine in the field of CML.

In a previous study, we showed that SOCS3 was important for lineage commitment of hematopoietic stem cells to erythroid cells. SOCS3 knock-down increased the expression of multiple erythroid-specific genes and inhibited the expression of genes controlling lymphoid differentiation [12]. In this study, we wanted to investigate the contribution of SOCS3 in pathogenesis of CML and further understand the potential underlying mechanisms of SOCS3. Previous studies suggested dysregulation of microRNA (miRNA) networks had been implicated in hematological malignancies, for example miR-29a/29b dysregulation played an important role in myeloid leukemogenesis [13]. So, understanding of miRNA biology in carcinogenesis could possibly pave novel routes for anti-cancer therapy [14, 15]. Here, we found that over-expression of SOCS3 in CML cells induced a transcriptional program enriched for leukemia suppression factors, including some miRNAs. For example, miR-124-3p was obviously up-regulated by SOCS3 over-expression. In turn, alterations of miR-124-3p expression levels influenced the effect of SOCS3 on CML cells. Furthermore, we confirmed that B4GALT1, a multidrug resistance gene, was the target gene of the SOCS3/miR-124-3p axis. These findings suggested the presence of a dysregulated molecular network involving SOCS3, miR-124-3p, and B4GALT1, which may provide novel insights into tumor biology and present a useful target for therapeutic interference of CML under certain circumstances.

Results

Expression of SOCS3 was dysregulated in CML cells

We measured the expression of SOCS3 by qPCR in a panel of human leukemia cell lines and primary bone marrow mononuclear cells (BMNCs) from healthy donors ($n = 3$). We found that SOCS3 expression was down-regulated in human leukemia cells, with the lowest expression levels in K562 cells, indicating that SOCS3 could be down-regulated in CML (Fig. 1a). We further analyzed the expression of SOCS3 in BMNCs

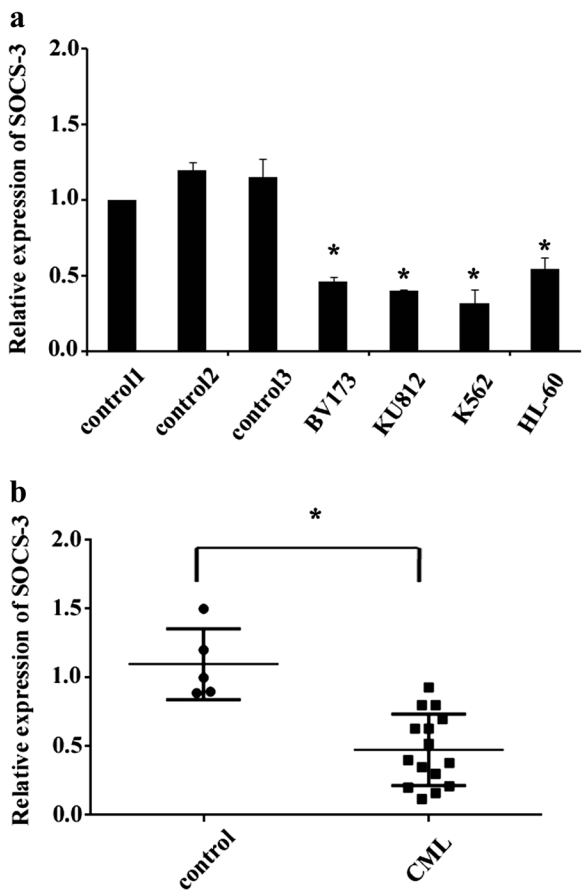

Fig. 1 Expression of SOCS3 was dysregulated in CML cells. **a** Expression of endogenous SOCS3 was examined by q-PCR in leukemia cell lines and BMNCs from three healthy donors. β-Actin was served as an internal control. SOCS3 expression in cells from donor 1 was set as a standard. Each point represented the mean of three independent experiments ($n = 3$).*$P < 0.05$; compared with standard. **b** SOCS3 expression in BMNCs from five healthy donors and 15 CML patients was examined by q-PCR. β-Actin was served as an internal control. SOCS3 expression in healthy donor 1 was set as a standard. Each point represented the mean of three independent experiments ($n = 3$). *$P < 0.05$; compared with the healthy donor group. All data were expressed as the mean ± SD

from 15 untreated CML patients. We found that compared with healthy donors ($n = 5$), SOCS3 expression was significantly down-regulated (which is below the minimal level of five healthy volunteers) in most of BMNCs from CML patients (12 of 15) (Fig. 1b). The protein expression of SOCS3 was also reduced greatly in CML cell lines and many patients (Additional file 1). All these findings suggested that expression of SOCS3 was dysregulated in CML cells.

SOCS3 regulated the growth of CML cells

To investigate the role of SOCS3 in CML cells, the expression and inference vectors of SOCS3 were stably transduced into K562 and KU812 cells using the lentiviral system. We found that SOCS3 over-expression induced a marked reduction in the number of colonies and SOCS3 knock-down led to a significant increase in the number of colonies from K562 cells by the clonogenic formation assay (Fig. 2a, b). Similar results were observed in KU812 cells (Fig. 2b). The CCK-8 assay confirmed that SOCS3 over-expression markedly inhibited the growth of K562 and KU812 cells, while SOCS3 down-regulation promoted the proliferation of these cells when compared with empty or non-targeting shRNA (shControl) groups (Fig. 2c).

SOCS3 contributed to imatinib-induced apoptosis of CML cells

We examined SOCS3 expression levels in K562 and KU812 cells prior to and after imatinib treatment. qPCR assays demonstrated a marked increase in mRNA levels of SOCS3 after imatinib treatment in two CML cell lines. Western blot assays confirmed a marked increase in the protein levels of SOCS3 after imatinib treatment (Fig. 3a). We also found that SOCS3 itself could not induce an obvious increase in apoptotic cells when K562 or KU812 cells were transduced with SOCS3 over-expression vector. However, SOCS3 over-expression could induce a marked increase in the percentage of apoptotic cells in the presence of imatinib (Fig. 3b, c).

SOCS3 inhibited the expression of leukemia-specific genes and promoted a series of miRNAs

To characterize the transcriptional changes caused by SOCS3, the gene expression profile of K562 was determined 48 h after SOCS3 over-expression or empty vector transduction. Total RNA from these cells was hybridized to an Illumina Human HT-12 v4 expression bead array. After normalization, the gene expression profile of two groups was compared using Benjamini Hochberg FDR values. Ultimately, we identified 296

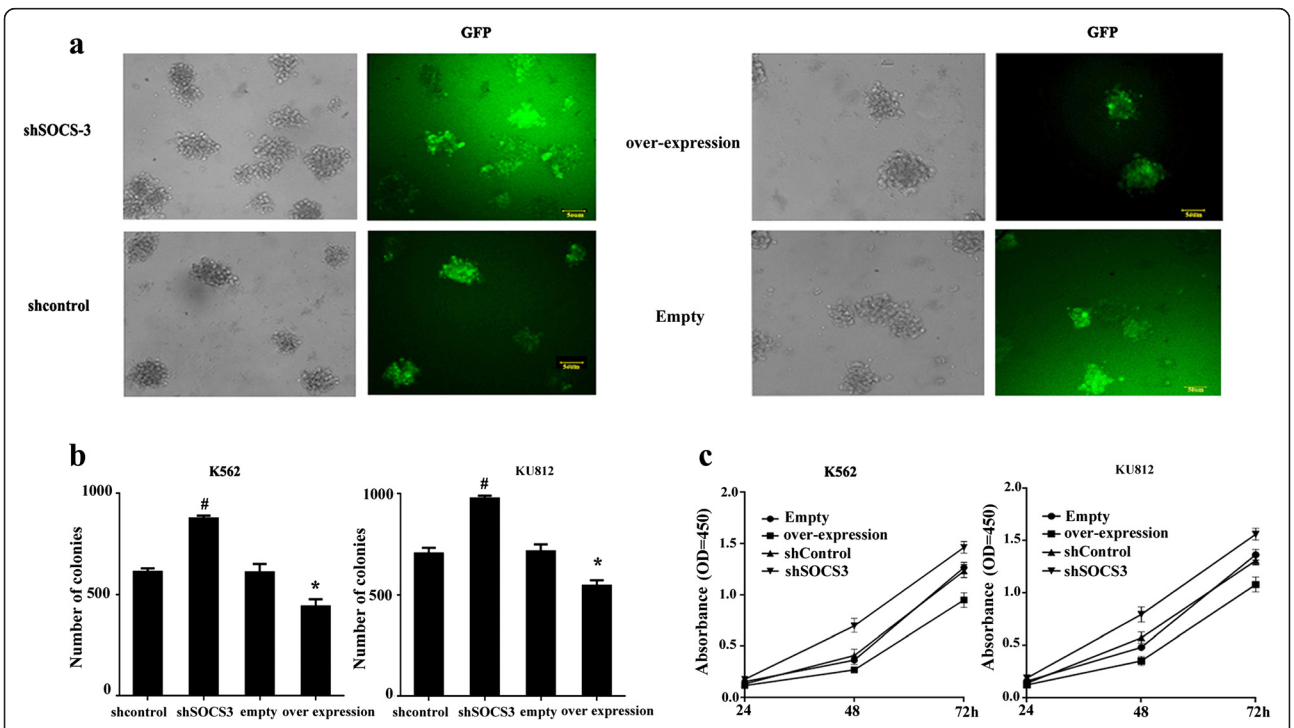

Fig. 2 SOCS3 regulated the proliferation and clonogenic growth of CML cells. **a** Representative pictures showing the effects of SOCS3 over-expression or knock-down on the clonogenic growth of K562 cells. Bar = 50 μm. **b** Statistical analysis for the number of colonies from CML cells after over-expression of SOCS3 or shRNA vector were transduced into K562 and KU812 cells. Data represented the mean ± SD from three experiments. **c** The effect of SOCS3 on cell proliferation was examined by the CCK-8 assay. Data represented the mean ± SD from three experiments ($n = 3$). $^{*}P < 0.05$; compared with the empty; $^{\#}P < 0.05$; compared with shControl

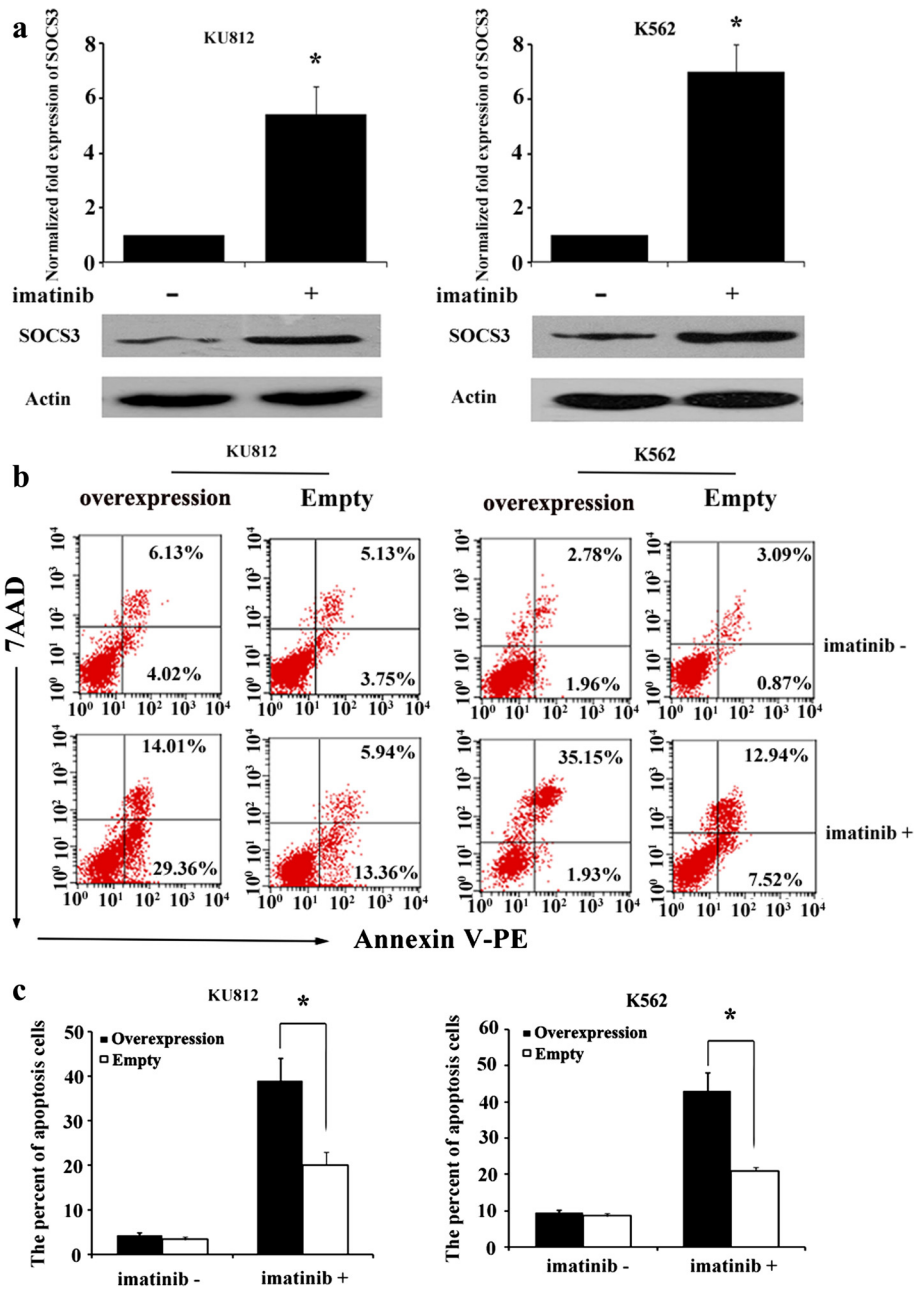

Fig. 3 SOCS3 was up-regulated by imatinib. **a** The expression of SOCS3 in K562 and KU812 cells was examined by qPCR and western blotting after they were treated with imatinib for 48 h. β-Actin was served as an internal control. Each value was the mean ± SD of three experiments ($n = 3$), *$P < 0.05$; compared with untreated cells. **b** Apoptosis of K562 and KU812 cells, which were transduced with SOCS3 over-expression or empty vectors, were examined by Annexin V staining following treatment with imatinib (48 h). **c** Statistical analysis of apoptosis in K562 and KU812 cells after imatinib treatment. Data represented the mean ± SD from three experiments ($n = 3$). *$P < 0.05$; compared with cells transduced with empty vector

genes with significant differential expression in K562 cells that over-expressed SOCS3. Among them, 198 genes were up-regulated and 98 genes were down-regulated. The pathways and genes that significantly changed were classified as follows: cancer pathways (*EPAS1*, *CCND1*, *FGF13*), myeloid leukemia pathways (*ZBTB16*, *KIT*, *IL13)*, hematopoietic cell lineage pathways (*EPOR*, *GYPA*, *CD36*) and so on (Fig. 4). In addition, we also found that some miRNAs were significantly affected by SOCS3 over-expression and that the expression of miR-124-3p was the highest in these miRNAs (data not shown).

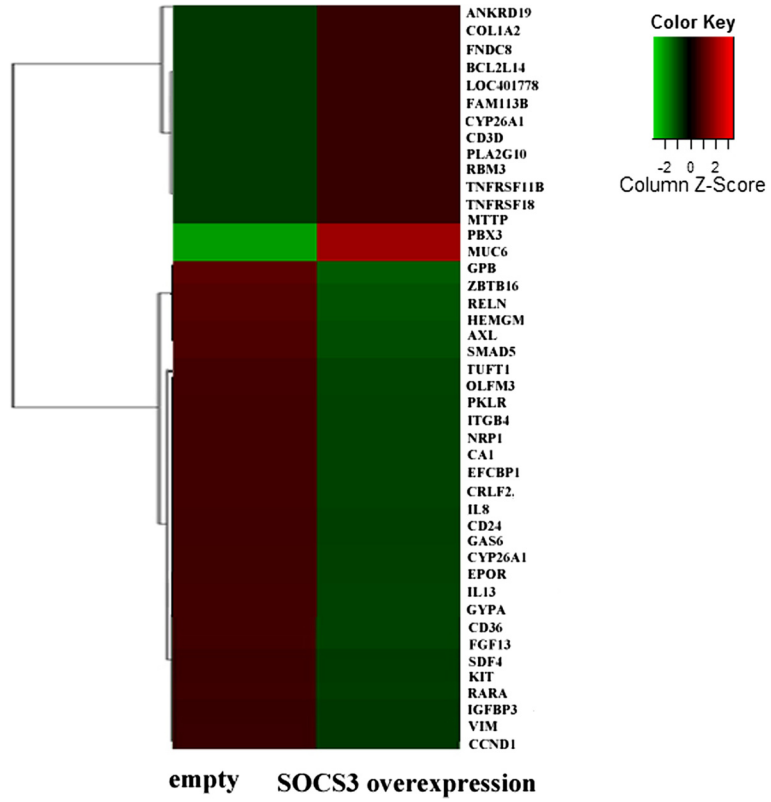

Fig. 4 The effect of SOCS3 expression on the gene expression profile of K562 cells. Heat-map showing gene expression profiles in K562 cells 48 h after transduction with SOCS3 over-expression or empty vectors. Each kind of sample was repeated three times by microarray ($n = 3$)

Next, we investigated SOCS3-induced gene expression alteration in K562 cells using GO analysis (Additional file 2). The results confirmed that the cancer and hematopoietic cell development signaling pathways were mainly associated with responses to over-expression of SOCS3 in K562 cells.

SOCS3 promoted miR-124-3p expression in CML cells

We further confirmed the effect of SOCS3 on the expression of miR-124-3p in K562 and KU812 cell lines by q-PCR. The results showed that SOCS3 over-expression enhanced the expression of miR-124-3p, and that SOCS3 knock-down inhibited the expression of miR-124-3p in both cell lines (Fig. 5 a, b). In addition, imatinib treatment resulted in a significant increase of miR-124-3p in CML cell lines, while up-regulation of miR-124-3p induced by imatinib was inhibited by SOCS3 knock-down in K562 and KU812 cells (Fig. 5c).

Next we explored the correlation between SOCS3 and miR-124-3p in BMNCs from CML patients ($n = 30$). The levels of SOCS3 and miR-124-3p were measured and normalized. As shown in Fig. 5d, when the relative expression levels of miR-124-3p were plotted against that of SOCS3 in each patient, a significant positive correlation was found (miR-124-3p vs. SOCS3: $R = 0.86$, $P < 0.05$).

We next investigated whether SOCS3 regulated CML cell function by up-regulating miR-124-3p. MiR-124-3p inhibitor and negative control were transduced into K562 and KU812 cells that were stably transduced by SOCS3 over-expression vector. Growth capacity was compared with cells transduced with empty expression vector. As expected, the cell proliferation assay and clonogenic assay showed that the miR-124-3p inhibitor partially neutralized the inhibiting effects of SOCS3 (Fig. 6).

B4GALT1 is a target of miR-124-3p in CML cells

Next, we searched for potential genes regulated by miR-124-3p in leukemogenesis. Using TargetScan and miRanda online search programs, we identified B4GALT1 as a potential target of miR-124-3p. A matched sequence was found at the nts 2089–2096 region of B4GALT1 mRNA 3'UTR (Fig. 7a).

To confirm that miR-124-3p targets the 3'UTR region of B4GALT1 in CML cells, HEK293 cells were co-transduced with miR-124-3p expression or control vector along with either the full-length 3'UTR of B4GALT1(Luci-B4GALT1) or mutated Luci-B4GALT1

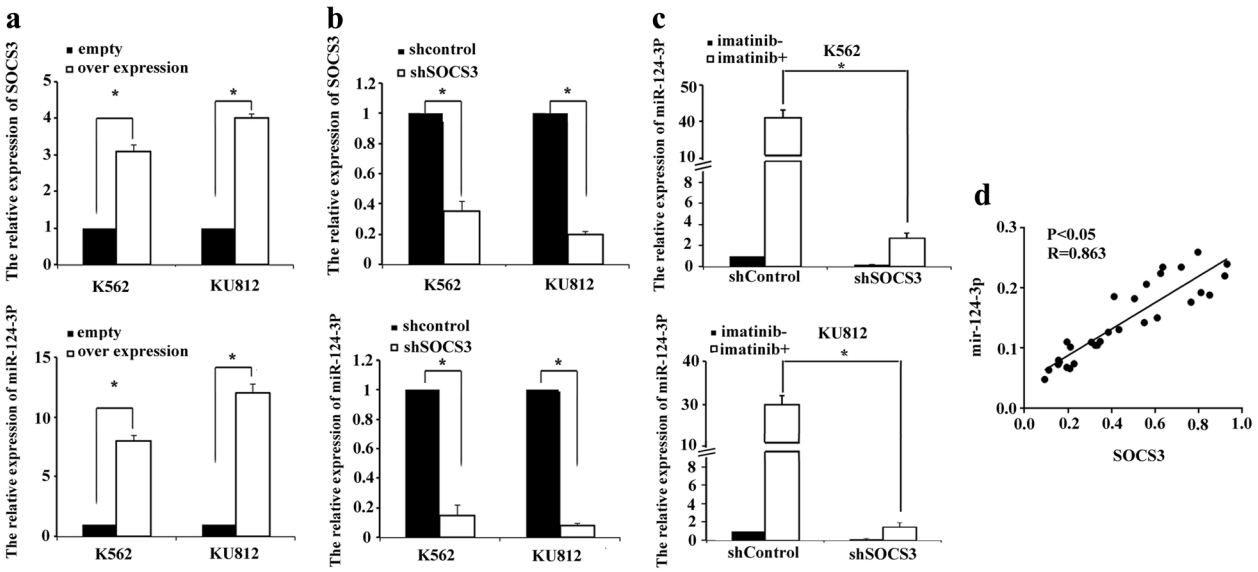

Fig. 5 SOCS3 regulated the expression of miR-124-3p in CML cells. The relative mRNA levels of SOCS3 or miR-124-3p in K562 and KU812 cells were analyzed by q-PCR after SOCS3 over-expression (**a**) or knock-down (**b**). Each value was the mean ± SD of three experiments ($n = 3$),*$P < 0.05$; compared with standard. **c** Relative mRNA levels of miR-124-3p in K562 and KU812 cells that were stably transduced with shSOCS3 or shControl vector were examined at 48 h following imatinib treatment. Results were normalized to untreated cells. RNU6-2 was served as an internal control. Each value was the mean ± SD of three experiments ($n = 3$), *$P < 0.05$; compared with shControl. **d** Statistically significant correlation between miR-124-3p and SOCS3 expression was observed by Pearson's method. The *y*-axis or *x*-axis represented the relative mRNA levels of miR-124-3p or SOCS3 in BMNCs from 30 CML patients, which were normalized against internal control RNU6-2 or β-Actin. Each point represented the mean of three independent experiments ($n = 3$). Data are expressed as the mean ± SD

reporter vectors bearing deletions of the 3'UTR target regions (△Luci-B4GALT1) (Fig. 7b). We found that luciferase activity of HEK293 cells was significantly decreased after co-transduction of miR-124-3p expression vector and a 3'UTR vector containing the B4GALT1/miR-124-3p target sequence (Fig. 7c).

Moreover, we over-expressed or inhibited the expression of miR-124-3p in K562 and KU812 cells and determined the endogenous expression of B4GALT1 at both the protein and mRNA level. We found that the mRNA level of B4GALT1 was not significantly affected by miR-124-3p in comparison with the control in both K562 and KU812 cells (Fig. 7d, e). However, B4GALT1 protein was markedly reduced after transduction with miR-124-3p expression vector, and vice versa (Fig. 7f). These data indicated that B4GALT1 was the target gene of miR-124-3p in CML cells, and miR-124-3p suppressed B4GALT1 gene expression at the post-transcriptional level.

B4GALT1 was regulated by the SOCS3/miR-124-3p axis

The expression of B4GALT1 protein was examined in K562 cells after the expression or inference vectors of SOCS3 were stably transduced into them. The results showed that B4GALT1 expression was inhibited by SOCS3 over-expression and promoted by SOCS3 knock down in vitro (Fig. 8a). We further found that SOCS3-induced down-regulation of B4GALT1 was attenuated by the presence of the miR-124-3p inhibitor (Fig. 8b).

To investigate the impact of B4GALT1 on SOCS3-induced chemo-sensitivity promotion in leukemia cells, K562 cells which stably over-expressed SOCS3 were transduced with the B4GALT1 expression vector or its negative control and then we examined apoptosis ratios of K562 cells in different groups after imatinib treatment. We found that ectopic expression of B4GALT1 significantly abrogated increasing chemo-sensitivity induced by SOCS3 over-expression in K562 cells (Fig. 8c).

Furthermore, K562 cells (5×10^6), which were transduced with SOCS3 over-expressing or empty vector, were inoculated into 25-g male nude mice subcutaneously ($n = 8$). The tumor formations were observed 4 weeks after inoculation. We found the weight of tumors in the SOCS3 over-expression group was significantly lower than that of the empty vector groups (Fig. 8d). Finally, tumor tissues were collected and sectioned. Immuno-histological examination was carried out using an antibody raised against B4GALT1. Fewer B4GALT1-positive cells were observed in the tumors of SOCS3 over-expressing group (Fig. 8e).

Discussion

In this study, we explored the function and involved mechanisms of SOCS3 in the pathophysiology of CML.

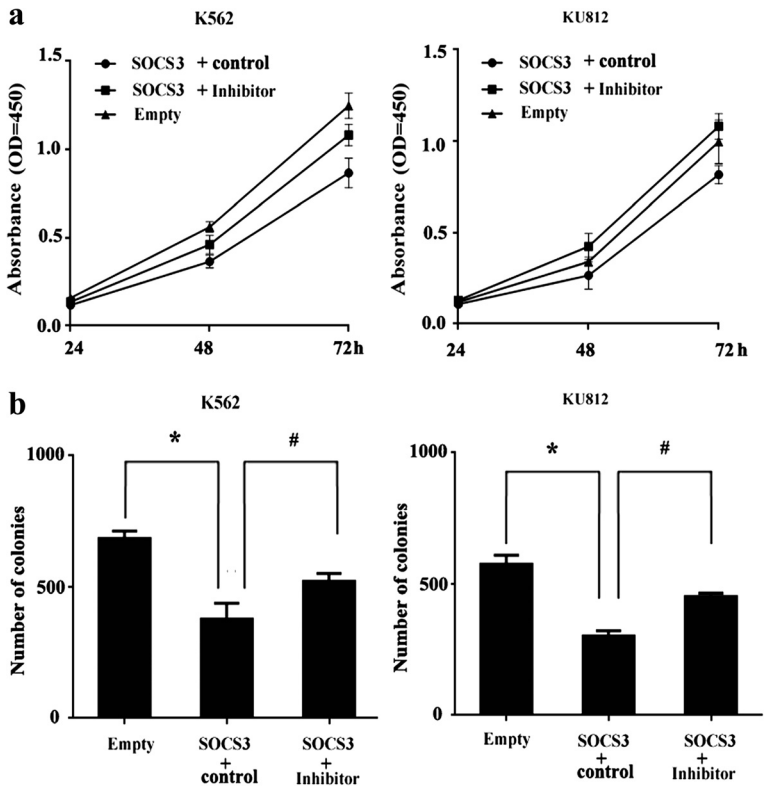

Fig. 6 miR-124-3p inhibitor partially neutralized the inhibitory effects of SOCS3. The miR-124-3p inhibitor and control were transduced into K562 and KU812 cells that were stably transduced with SOCS3 over-expression vector. The effects of miR-124-3p on cell growth (**a**) and colony formation (**b**) were determined. Data represented three independent experiments and were shown as the mean ± SD ($n = 3$), *$P < 0.05$; compared with empty vector or inhibitor

Firstly, we found that both mRNA and protein expression of SOCS3 were down-regulated in CML cell lines and most of samples from CML patients. Sakai et al. observed a large variation of SOCS3 expression in different patients [16]; here, we also found the significant difference of SOCS3 expression in different patients which implied SOCS3 may be used as an index for CMLprecision diagnosis in the future.

Previous studies demonstrated that the expression of SOCS3 was associated with the response of CML cells to IFN-alpha [16, 17], and down-regulation of SOCS3 was a possible reason for imatinib resistance of leukemia cells [11]. Consistently, in our study, the expression of SOCS3 in CML cells was up-regulated after imatinib treatment. Although SOCS3 exerted no remarkable effect on apoptosis, SOCS3 over-expression could enhance imatinib-induced apoptosis in CML cells. Takeuchi et al. suggested increased expression of SOCS3 in bone marrow cells may result from the action of several cytokines secreted in the bone marrow environment [17], so we speculated the bone marrow environment alteration caused by imatinib treatment may induce the up-regulation of SOCS3 in CML cells here. However, the in-depth mechanism needed to be clarified.

miRNAs play important roles in tumorigenesis [18–23]. We then explored whether SOCS3 regulated the growth and chemo-sensitivity of CML cells by modulating miRNA. The gene expression bead array results indicated that miR-124-3p, a tumor suppressor [24–26], was significantly affected by SOCS3. Fowler et al demonstrated that over-expression of miR-124 in GBM cells was associated with diminished tumor cell migration and invasion [27]. Moreover, Shi et al. found that miR-124-3p could inhibit the proliferation of prostate cancer cells [28]. In our study, we found that miR-124-3p expression in CML cell lines was regulated by SOCS3. A significant positive correlation between miR-124-3p and SOCS3 was observed. And, the inhibitory effect of SOCS3 on CML cell proliferation was attenuated in the absence of miR-124-3p. All these data indicated that miR-124-3p play an important role in SOCS3-mediated growth inhibition.

Recent study demonstrated that B4GALT1 gene family play an important role in the resistance of human leukemia cells to therapeutic drugs. In multidrug resistance leukemia patients, highly expressed B4GALT1 regulated the hedgehog pathway, and were associated with the expression of p-glycoprotein and multidrug resistance-

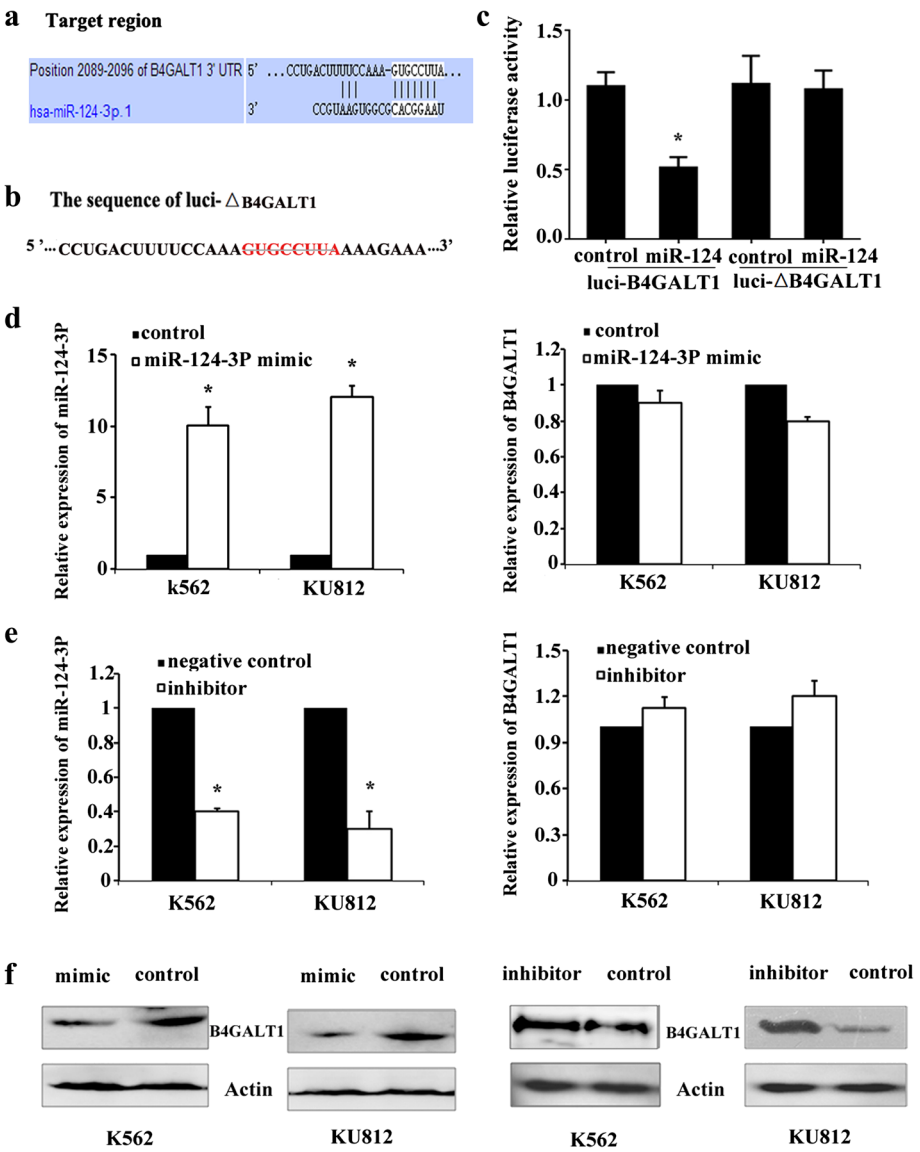

Fig. 7 B4GALT1 was a target of miR-124-3p. **a** Bioinformatics analysis of the predicted interactions of miR-124-3p and its binding sites within the 3'UTR of B4GALT1. **b** Mutated sequences used in the luciferase assay. **c** Luciferase analysis in HEK293 cells. The expression levels of miR-124-3p or B4GALT1 in K562 and KU812 cells were analyzed by qPCR after they were transduced with miR-124-3p expression mimic (**d**) or inhibitor (**e**). U6 or β-Actin was served as an internal control. Each value was the mean ± SD of three experiments ($n = 3$),*$P < 0.05$; compared with control or negative control. **f** Western blot analysis of B4GALT1 expression in K562 and KU812 cells after they were transduced with miR-124-3p expression mimic or miR-124-3p inhibitor for 48 h. β-Actin was served as an internal control ($n = 3$)

associated protein, resulting in the specific drug-resistant phenotypes of leukemia cell lines [29, 30]. Here, we first demonstrated that B4GALT1 was a target gene of miR-124-3p as predicted by bioinformatics, verified the conserved region in the B4GALT1 3'UTR was binding to miR-124-3p. In addition, we found that B4GALT1 protein expression was significantly down-regulated by miR-124-3p over-expression in CML cells. So, we speculated that SOCS3 enhanced the chemo-sensitivity of CML cells by down-regulating B4GALT1, and that miR-124-3 was the link between them. Thus, we analyzed the effect of SOCS3 on the expression of B4GALT1 and proved that SOCS3 modulated the expression of B4GALT1 by miR-124-3p and, in turn, B4GALT1 could rescue SOCS3-induced chemo-sensitivity alterations in K562 cells. Finally, tumorigenicity assays in nude mice confirmed that over-expression of SOCS3 inhibited the proliferation of K562 cells and down-regulated the expression of B4GALT1 in vivo. However, the in vivo function of SOCS-3 after imatinib treatment needs to be investigated in the further study.

Our results showed SOCS-3 regulated miR-124-3p/B4GALT1 pathway played an important role in the

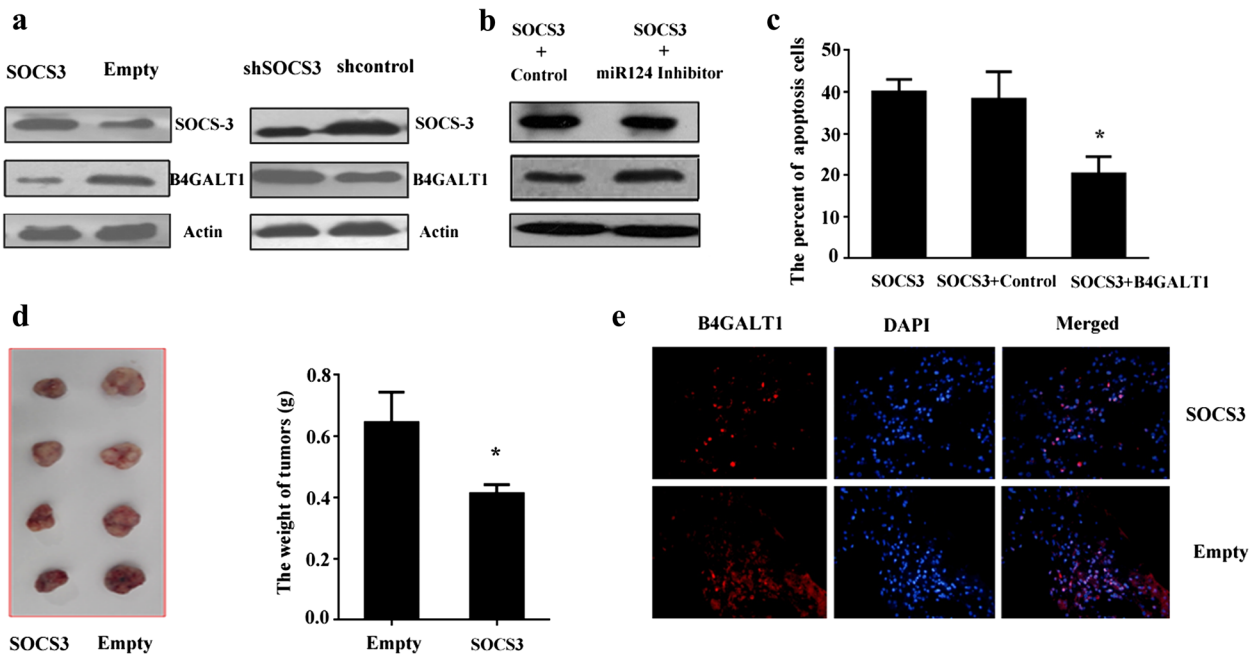

Fig. 8 B4GALT1 was regulated by SOCS3/miR-124-3p axis. **a** Western blot analysis of B4GALT1 expression in K562 cells after SOCS3 up- or down-regulation. β-Actin was served as an internal control ($n = 3$). **b** Western blot analysis of B4GALT1 expression in K562 cells stably expressing SOCS3 after transduction with the miR-124-3p inhibitor or negative control. β-Actin was served as an internal control ($n = 3$). **c** Over-expression of B4GALT1 rescued SOCS3-induced chemo-sensitivity promotion. B4GALT1 expression and control vector were transduced into K562 cells that over-expressed SOCS3. Apoptosis ratios of K562 cells in different groups were examined by Annexin V assay after imatinib treatment. Data represent the mean ± SD from three experiments ($n = 3$). **d** SOCS3 over-expression inhibited tumor growth of K562 in vivo. *$P < 0.05$; compared with empty group. Each point represented the mean of three independent experiments ($n = 3$). Data are expressed as the mean ± SD. **e** SOCS3 over-expression inhibited the expression of B4GALT1 in vivo. B4GALT1 expression in tumors from K562 cells was analyzed by immunohistochemistry

pathogenesis of CML. However, imatinib treatment still induced miR-124-3p increase in the absence of SOCS3 and SOCS3 could inhibit colony formation, regardless of the presence of miR-124-3p inhibitor, which implied other signal pathways may be involved. For example, previous studies demonstrated JAK/STAT pathway and cytokine signal pathways were involved in SOCS3-mediated effects in CML cells [16, 17].

Resistance to targeted drugs remains a challenge for CML therapy. Accurate biomarkers are of great importance to antitumor therapeutics [31]. In this study, there was an obvious correlation between SOCS3 expression and the sensitivity of CML cell lines to imatinib. Thus, SOCS3 may be used as a novel biomarker predicting the response to targeted drugs and it was of great value to further elucidate the role and mechanism underlying SOCS3 expression in CML cells. However, we did not explore the role of SOCS3 in primary cells from CML patients who are resistant to imatinib, and we acknowledge this is a limitation of this study.

Conclusions

In summary, our work revealed that an interesting signal pathway initiated by SOCS3 was involved in CML development. Down-regulated SOCS3 in CML cells was associated with low level of miR-124-3p, then could not exert enough repressive effect on B4GALT1, resulting in the proliferation of CML cells and targeted drugs resistance. In conclusion, SOCS3/miR-124-3p/B4GALT1 signaling pathway plays an important role in the pathophysiology of CML. SOCS3 may be used as an index for CML diagnosis, and a novel biomarker predicting the response of CML to targeted drugs, in clinical settings.

Methods

Patient samples

BMNCs were obtained from patients with confirmed diagnose of CML and from healthy donors with informed consent. BMNCs were enriched by Ficoll gradient centrifugation. The study was performed with the approval of the Ethics Committee of Chinese PLA General Hospital, Beijing, China.

Cell culture

K562, KU812, HL-60, and BV173 cells were cultured in RPMI-1640. HEK293 cells were cultured in DMEM. These mediums contained 10 % (v/v) fetal bovine serum (Gibco, Life technologies, USA) and 100 mg/mL

penicillin/streptomycin. For imatinib treatment, cells were treated with 1 μmol/L imatinib for 48 h.

Lentiviral plasmid construct and transduction

Lentiviral expression and interference vectors targeting human SOCS3 were constructed as described previously [12]. Empty expression vector (empty) or non-targeting siRNA (shControl) were used as controls of expression and interference vectors, respectively. Lentiviral particles were produced and cells were transduced. After transduction (48 h), positive cells were sorted by fluorescence-activated cell sorting (FACS), according to the expression of green fluorescent protein (GFP).

Quantitative RT-PCR

Total RNA isolation, reverse transcription, and the quantification of target gene expression were performed as previously described [12]; β-Actin was used as an internal control. miR-124-3p expression levels were quantified using U6 as the internal control (GenePharm). The fold-change in expression was calculated using the following primer pairs for the amplification of target mRNAs: SOCS3 forward primer 5'ATCCTGGTGACAT GCTCCTC'3 and reverse primer 5'CAAATGTTGCTTCCCCCTTA'3; β-Actin forward primer 5' GATCCACATCTGCTGGAA GG'3 and reverse primer 5'AAGTGTGACGTT GAC ATCCG'3; B4GALT1 forward primer 5'AACCATGT GACTGAGTGC CC'3 and reverse primer 5'TCAG TGTGTTGTGCCAAAGC'3; Micro124-3p forward primer 5'TAAGGCACGCGGTGAATGCC'3 and reverse primer 5'GATTGAATCGA GCACCAG TTAC'3; U6 forward primer 5'CGCTTCGGCAG CAC ATATACTA'3 . Unified reverse primer 5'GATTGAATCGA GCACCAG TTAC'3.

Western blot analysis

Cells were lysed directly in lysis buffer to collect whole cell extracts. Protein samples were separated on polyacrylamide gels, transferred onto nitrocellulose membrane by iblot (Invitrogen), detected using horseradish peroxidase-conjugated secondary antibodies, and exposed to BioMax film (Kodak) following chemiluminescence (Santa Cruz, CA, USA). The following primary antibodies were used: SOCS3, Actin, and B4GALT1 (Santa Cruz, CA, USA).

Cell proliferation assay

Cell proliferation was determined using CCK-8 (Dojindo, Japan) method. K562 cells (3000 cells/well) were plated in 96-well plates. At different time points, CCK-8 reagents were added to each well and further incubated at 37 °C for 2 h. The number of viable cells was assessed by measurement of absorbance at 450 nm using a Multiskan (Thermo Scientific, Asheville, NC, USA).

Clonogenic progenitor cell assay

Cells were seeded in a 6-well plate with methylcellulose medium (MethoCult H4435, STEMCELL Technologies, Canada) according to the manufacturer's instructions. After 1 week of cultivation, colonies were counted.

Apoptosis assay

The apoptosis assays were performed using the Annexin V-PE kit (BioLegend, San Diego, CA, USA) according to the manufacturer's protocol. The stained cells were immediately analyzed on a FACScalibur flow cytometer (Becton Dickinson). The data were expressed as the percentage of apoptosis cells.

Whole-genome expression analysis

Total RNA was extracted from K562 cells transduced with over-expression SOCS3 or empty vector (5×10^6 cells) using Trizol reagent (Invitrogen Life Technologies, Paisley, UK). Genome expression analysis was performed by Illumina Human HT-12 v4 BeadChip (Illumina, San Diego, CA, USA) at the Beijing Qian zhao xing ye Biological Technology Co., Ltd. (Beijing, China).

miR-124-3p mimic and inhibitor

The hsa-miR-124-3p mimic or control sequence, and hsa-miR-124-3p inhibitor and hsa-miR-124-3p inhibitor negative control were all purchased from GenePharma (Shanghai, China).

Luciferase assays

The human pre-miR-124 sequence was amplified and cloned into pcDNA3.1 constructs (Invitrogen) to generate the pcDNA3.1-miR-124 expression vector. The full-length 3'UTR of B4GALT1 was amplified using cDNA from K562 cells and double-digested with XbaI/EcoRI and cloned downstream of the firefly luciferase coding region sites of a modified pGL3-control plasmid named Luci-B4GALT1. We also constructed mutated Luci-B4GALT1 reporter vectors bearing deletions of the UTR target regions and named them Luci-△B4GALT1. The vectors were co-transduced with control or pcDNA3.1-miR-124 expression vectors into K562 cells. Lysates were prepared 48 h after transduction. Luciferase activity was measured using a dual-luciferase reporter assay system (Promega). All experiments were performed in triplicate at least three independent times.

B4GALT1 expression plasmid construction and transduction

The full-length of the human B4GALT1 sequence was amplified and cloned into the pcDNA3.1 vector to generate the B4GALT1 expression vector. K562 cells were transduced with the B4GALT1 expression vector in 24-well plates using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Tumorigenicity assays in nude mice

A total of 5×10^6 K562 cells that were stably transduced with SOCS3 over-expression or empty vectors were injected into male nude mice subcutaneously ($n = 8$). Mice were sacrificed at 28 days post-inoculation, and the tumors were excised and their weight was measured and photographed. Tumors from different groups were removed and fixed with formaldehyde, embedded in paraffin wax, and sectioned. The sections were cleared through xylene, graded ethanol, water and incubated with anti-B4GALT1 antibodies (Santa Cruz) at 4 °C overnight, stained with DAPI, washed with PBS three times, and observed by fluorescence microscopy.

Statistical analysis

All data were expressed as the mean ± standard deviation (SD). The differences between two groups were assessed by Student's t test. Pearson's correlation coefficient was calculated to analyze the correlation. $P < 0.05$ was considered to be statistically significant.

Additional files

Additional file 1: Figure S1. Expression of SOCS3 protein in CML cells. SOCS3 expression in CML cell lines and BMNCs from CML patients was analyzed by Western blotting. β-Actin was served as an internal control ($n = 3$). (TIF 69 kb)

Additional file 2: Figure S2. GO analysis of SOCS3-induced gene expression alteration in K562 cells. Branches of the GO hierarchical tree with significantly enriched GO terms were indicated in red boxes. Insignificant GO terms within the hierarchical tree are shown as white boxes. (TIF 203 kb)

Abbreviations

3′-UTR, 3′-untranslated region; BMNCs, bone marrow mononuclear cells; CML, chronic myeloid leukemia; CMPD, chronic myeloproliferative disorders; FACS, fluorescence - activated cell sorting; GFP, green fluorescent protein; miR-124-3p, microRNA-124-3p; miRNAs, microRNAs; qPCR, quantitative real-time PCR; siRNA, small interfering RNA; SOCS3, suppressor of cytokine signaling 3; WB, Western blotting

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Availability of data and materials

The dataset supporting the conclusions of this article are included within the article and its additional file.

Authors' contributions

YXL, ZZH, JHX, and CJG participated in designing the research. YXL and LW performed the research and data analysis. WJL and HTZ performed the luciferase reporter assay experiments. LW and CJG contributed the patient samples and participated in their characterization. WJL performed tumorigenicity assays. YXL wrote the paper. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

RNA from patients was isolated from the peripheral blood of CML patients after written informed consent to research studies, and the study was approved by the Ethics Committee of Chinese PLA General Hospital. All animal experiments were conducted in agreement with the Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of the Chinese PLA General Hospital.

Author details

[1]Department of Neurosurgery, The First Affiliated Hospital of Chinese PLA General Hospital, 51 Fushi Road, Beijing 100048, People's Republic of China. [2]Department of Hematology, Chinese PLA General Hospital, Laoshan Branch, No. 401, Qingdao 266100, People's Republic of China. [3]Department of Hematology, Chinese PLA General Hospital, 28 Fuxing Road, Beijing 100853, People's Republic of China. [4]Genetic Laboratory of Development and Diseases, Beijing Institute of Biotechnology, Beijing 100071, People's Republic of China.

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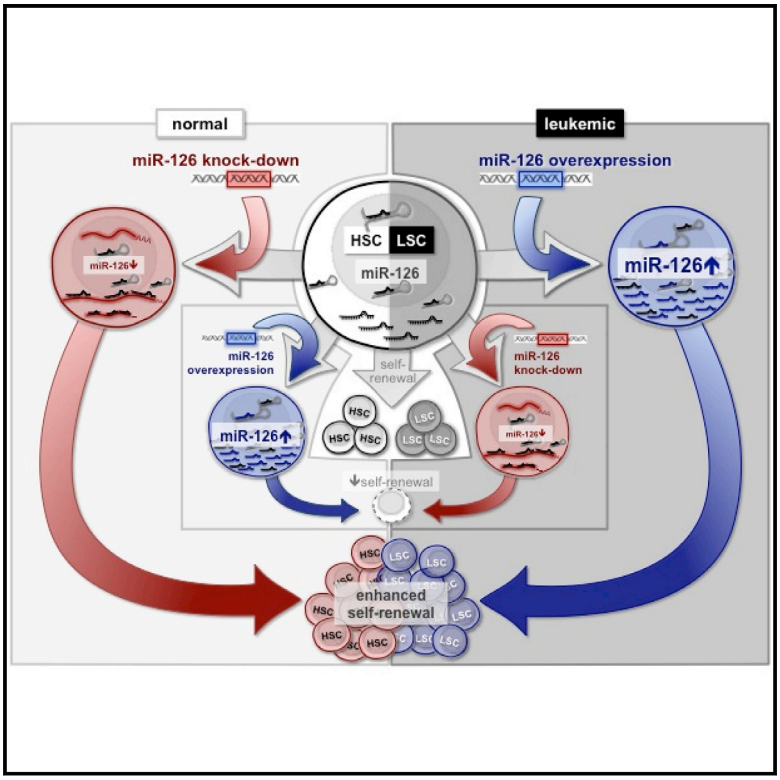
Cancer Cell

miR-126 Regulates Distinct Self-Renewal Outcomes in Normal and Malignant Hematopoietic Stem Cells

Graphical Abstract



Authors

Eric R. Lechman, Bernhard Gentner, Stanley W.K. Ng, ..., Jean C.Y. Wang, Luigi Naldini, John E. Dick

Correspondence

jdick@uhnresearch.ca

In Brief

Lechman et al. show that miR-126 targets the PI3K/AKT/MTOR signaling pathway to preserve quiescence, increase self-renewal, and promote chemotherapy resistance of acute myeloid leukemia stem cells (LSC). Reducing the miR-126 level impairs LSC maintenance in contrast to expanding normal hematopoietic stem cells.

Highlights

- Clinical outcome in AML correlates with LSC-associated miRNA expression
- miR-126 targets multiple components of the PI3K/AKT/MTOR signaling pathway
- miR-126 promotes chemotherapy resistance by preserving LSC in a quiescent state
- miR-126 governs opposing self-renewal outcomes in normal and malignant stem cells

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miR-126 Regulates Distinct Self-Renewal Outcomes in Normal and Malignant Hematopoietic Stem Cells

Eric R. Lechman,[1,2,16] Bernhard Gentner,[3,4,5,16] Stanley W.K. Ng,[6,7] Erwin M. Schoof,[1,2] Peter van Galen,[1,2] James A. Kennedy,[1,8] Silvia Nucera,[3,4] Fabio Ciceri,[4,5] Kerstin B. Kaufmann,[1,2] Naoya Takayama,[1,2] Stephanie M. Dobson,[1,2] Aaron Trotman-Grant,[1,2] Gabriela Krivdova,[1,2] Janneke Elzinga,[1,2] Amanda Mitchell,[1,2] Björn Nilsson,[9] Karin G. Hermans,[1,2] Kolja Eppert,[10] Rene Marke,[11] Ruth Isserlin,[7] Veronique Voisin,[7] Gary D. Bader,[7] Peter W. Zandstra,[6,7] Todd R. Golub,[12] Benjamin L. Ebert,[13] Jun Lu,[14] Mark Minden,[1,8] Jean C.Y. Wang,[1,8] Luigi Naldini,[3,4] and John E. Dick[1,2,15,*]

[1]Princess Margaret Cancer Centre, University Health Network
[2]Department of Molecular Genetics
University of Toronto, Toronto, ON M5G 1L7, Canada
[3]San Raffaele Telethon Institute for Gene Therapy
[4]Vita Salute San Raffaele University, San Raffaele Scientific Institute
[5]Hematology and Bone Marrow Transplantation Unit
San Raffaele Hospital, Milan 20132, Italy
[6]Department of Chemical Engineering and Applied Chemistry, Institute of Biomaterials and Biomedical Engineering, University of Toronto, Toronto, ON M5G 2M9, Canada
[7]The Donnelly Centre, University of Toronto, Toronto, ON M5S 3E1, Canada
[8]Department of Medicine, University of Toronto, Toronto, ON M5G 2M9, Canada
[9]Department of Hematology and Transfusion Medicine, Lund University Hospital, Lund 221 84, Sweden
[10]Department of Pediatrics, McGill University and The Research Institute of the McGill University Health Centre, Montreal, QC H4A 3J1, Canada
[11]Laboratory of Pediatric Oncology, Radboud University Medical Center, Nijmegen, 6500 HB, Netherlands
[12]Department of Pediatric Oncology, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115, USA
[13]Division of Hematology, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA
[14]Yale Stem Cell Center, Yale Cancer Center, Yale University School of Medicine, New Haven, CT 06520, USA
[15]Princess Margaret Cancer Research Tower, Room 8-301, 101 College Street, Toronto M5G 1L7, Canada
[16]Co-first author
*Correspondence: jdick@uhnresearch.ca
http://dx.doi.org/10.1016/j.ccell.2015.12.011

SUMMARY

To investigate miRNA function in human acute myeloid leukemia (AML) stem cells (LSC), we generated a prognostic LSC-associated miRNA signature derived from functionally validated subpopulations of AML samples. For one signature miRNA, miR-126, high bioactivity aggregated all in vivo patient sample LSC activity into a single sorted population, tightly coupling miR-126 expression to LSC function. Through functional studies, miR-126 was found to restrain cell cycle progression, prevent differentiation, and increase self-renewal of primary LSC in vivo. Compared with prior results showing miR-126 regulation of normal hematopoietic stem cell (HSC) cycling, these functional stem effects are opposite between LSC and HSC. Combined transcriptome and proteome analysis demonstrates that miR-126 targets the PI3K/AKT/MTOR signaling pathway, preserving LSC quiescence and promoting chemotherapy resistance.

Significance

Leukemia stem cells play central roles in disease progression and recurrence due to their intrinsic capacity for self-renewal and chemotherapy resistance. However, few regulators of human LSC function are known. Our study establishes that miRNA plays a powerful role in governing the fundamental properties that define the stemness state of human LSC including quiescence, self-renewal, and chemotherapy response. Self-renewal regulators have remarkably parallel functions in malignant and normal stem cells, precluding their therapeutic targeting because of toxicity to normal stem cells. The opposing self-renewal outcomes governed by miR-126 within HSC and LSC indicate that despite shared stemness determinants, it may be possible to target therapeutically the networks that specifically control LSC through perturbation of miR-126 levels.

INTRODUCTION

Acute myeloid leukemia (AML) is organized as an aberrant developmental hierarchy maintained by functionally distinct leukemia stem cells (LSC) (Kreso and Dick, 2014). LSC are linked to therapy failure and disease recurrence, but they also share many biological properties with hematopoietic stem cells (HSC), including capacity for self-renewal and quiescence (Kreso and Dick, 2014). Several self-renewal regulators have been studied in both HSC and LSC contexts including PTEN, BMI1, GFI1, TEL1, STAT5, and JUNB; except for PTEN, loss of function typically impairs self-renewal of both LSC and HSC (Yilmaz and Morrison, 2008). LSC and HSC are both quiescent, although quiescence regulation is better understood in HSC. Several intrinsic and extrinsic signals converge upon cyclins and cyclin-dependent kinases (CDKs) that act upstream of Retinoblastoma (RB) family members to regulate early and late G_1 progression in HSC (Viatour et al., 2008), while the G_0 state is governed by MTORC1 and CDK6 (Laurenti et al., 2015; Rodgers et al., 2014). Quiescence and distinct G_0 exit kinetics are essential HSC properties (Trumpp et al., 2010). Although LSC quiescence is less well defined, the known regulators appear to function similarly in LSC and HSC, with LSC quiescence often invoked as a mechanism of chemotherapy resistance (Holtz et al., 2007). Additional studies are required to determine if differences exist in self-renewal and quiescence regulation between LSC and HSC and whether it is possible to develop therapies that eradicate LSC while sparing HSC.

Transcriptional analysis of human HSC and functionally defined LSC have defined stemness signatures that are highly prognostic for patient survival, establishing that LSC-specific properties are clinically relevant (Eppert et al., 2011; Metzeler et al., 2013). However, little is known of how stemness programs are controlled. Several differentially expressed miRNAs were identified and found to control HSC (Hu et al., 2015; Lechman et al., 2012; Mehta et al., 2015; O'Connell et al., 2010) by coordinate repression of multiple targets (Ebert and Sharp, 2012). In hematopoiesis, most miRNAs affect progenitor lineage commitment and mature cell function (Undi et al., 2013), although HSC self-renewal can be governed by miR-125a/b, miR-29a, and miR-126 (Ooi et al., 2010; O'Connell et al., 2010; Guo et al., 2010; Lechman et al., 2012). miR-126 plays a role, conserved in both human and mouse, in maintaining HSC quiescence by attenuating the cellular response to extrinsic signals via targeting multiple components of the PI3K/AKT/GSK3B signaling pathway (Lechman et al., 2012). Thus, HSC expand without concomitant exhaustion upon miR-126 silencing.

Deregulation of miRNAs occurs in leukemia correlating with known risk categories and prognosis (Garzon et al., 2008; Li et al., 2008; Marcucci et al., 2009). Functionally, miRNA overexpression can induce murine leukemic transformation (Han et al., 2010; O'Connell et al., 2010; Song et al., 2013). Several LSC-associated miRNAs are functional: miR-17-92 polycistron maintained LSC in MLL models (Wong et al., 2010), whereas antagonizing miR-196 and miR-21 reduced LSC in an experimental human MLL model (Velu et al., 2014). Targeted miR-126 reduction in cell lines and primary AML samples reduced AML growth, although mechanisms were not reported (Dorrance et al., 2015; de Leeuw et al., 2014). These promising studies point to the importance of further understanding the role of miRNA in governing stemness in AML. Here, we investigated the role of miR-126 in governing LSC self-renewal, quiescence, and chemotherapy resistance.

RESULTS

LSC miRNA Signature Is Prognostic for Patient Outcome

To determine whether miRNA are differentially expressed in LSC and HSC, we fractionated 16 AML patient samples and three lineage-depleted (Lin^-) cord blood (CB) samples using CD34 and CD38 into four populations and subjected each to global miRNA profiling; the stem cell content of each fraction was functionally assayed by xenotransplantation (Figures 1A and S1A). Bioinformatic analysis of 25 LSC-enriched and 27 fractions devoid of LSC activity (Figure S1A) revealed a human LSC-associated miRNA signature derived from in vivo functionally validated AML patient samples (Figure 1B). In parallel, miRNAs enriched in HSC or committed progenitors were determined (Figure S1B). By comparing similar immunophenotypic AML and normal populations, several differentially expressed miRNAs were found (Figure S1C).

To determine if the LSC-associated miRNA signature was clinically relevant, a regression analysis was performed on 74 AML patients with normal cytogenetics (PMCC cohort, Table S1). An optimized LSC signature consisting of four miRNAs was identified, each with differential weights based on impact upon overall survival (OS) (Figure 1C). This signature was prognostic of OS in both univariate (Figure 1D) and multivariate analyses (Figure 1E) in an independent cohort. Together with prior studies showing that LSC-specific gene expression signatures are significantly prognostic (Eppert et al., 2011; Greaves, 2011), these data establish that LSC properties influence clinical outcomes and that miRNAs play a powerful role in regulating LSC stemness.

miR-126 Bioactivity Enriches for LSC Activity

Further functional studies on AML focused on miR-126 as it is a known HSC regulator (Lechman et al., 2012). qPCR independently confirmed that LSC-containing AML fractions generally expressed the highest miR-126 levels (Figure S2A). As miRNA expression does not uniformly equate with miRNA bioactivity, a miR-126 lentiviral reporter vector was used to investigate whether miR-126 is biologically active in LSC (Gentner et al., 2010); ΔNGFR levels indicate transduced cells, while EGFP levels are inversely correlated with miR-126 bioactivity (Figure S2B). Four primary AML samples (Table S2) were transduced with the reporter, transplanted into xenografts, and after 12 weeks the engrafting population was sorted solely on the basis of miR-126 bioactivity (Figure 2A). Each sorted population was transplanted into secondary mice and LSC activity scored after 8 weeks, based on whether the engrafting population recapitulated the same EGFP/ΔNGFR flow profile as the primary recipient (a cardinal property of cancer stem cells). Despite the presence of LSC activity in multiple subpopulations with CD34 and CD38 sorting (Table S2), miR-126 bioactivity aggregated all LSC activity into a single $miR\text{-}126^{high}$ population (Figure 2B). qPCR confirmed 40-fold higher mature miR-126 levels in LSC-engrafting fractions compared with non-engrafting fractions for three AML samples (Figure S2C). LSC-containing fractions also had the highest clonogenic (Figure S2D) and proliferative

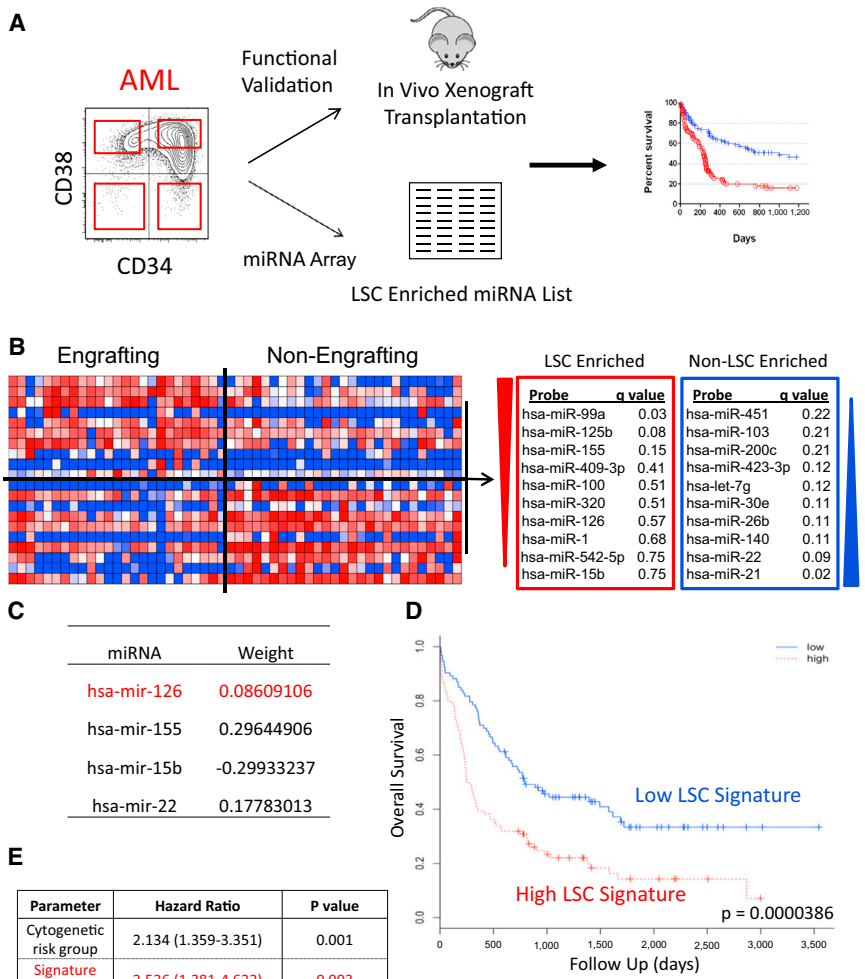


Figure 1. Generation and Validation of an LSC-Enriched miRNA Signature

(A) Schematic depicting the strategy to fractionate human AML patient samples based on immunophenotypic staining for CD34 and CD38. Functional validation of sorted fractions was performed by xenotransplantation, the result of which was combined with miRNA expression profiling to generate stem cell-related miRNA expression profiles.

(B) Heatmap and summary of miRNAs enriched within the LSC and non-LSC populations.

(C) The optimized miRNA signature derived from regression analysis of the PMCC cohort and the weight each miRNA adds to the overall signature.

(D and E) Validation of the optimized LSC-associated miRNA signature shown in (C) in the TCGA cohort (n = 187) by (D) univariate analysis (hazard ratio [HR], 2.04; $p < 0.0001$) and (E) multivariate analysis (HR, 2.53; $p = 0.003$). See also Figure S1 and Table S1.

potential (Figure S2E). These data indicate that miR-126 bioactivity is directly linked to LSC function and that it is possible to exploit miRNA bioactivity for prospective LSC isolation, circumventing often unreliable and heterogeneously expressed cell surface markers (Kreso and Dick, 2014).

Clinical Relevance of miR-126 Expression

To determine if miR-126 expression alone is prognostic, the PMCC cohort (Table S1) was investigated, and increased miR-126 expression was found to be associated with worse OS (median OS of 28.5 months [high expression] versus not reached [low expression]; Figure 2C), event-free survival (Figure 2D), and relapse-free survival (Figure 2E), a result in keeping with other studies (Dorrance et al., 2015; de Leeuw et al., 2014). Since miR-126 expression is high in patients with t(8; 21) and inv(16) (Li et al., 2008), we evaluated the prognostic value of miR-126 after excluding these patients from The Cancer Genome Atlas (TCGA) dataset. High miR-126 was associated with decreased survival in the TCGA dataset (median OS of 12.3 months [high expression] versus 18.5 months [low expression]; Figure 2F). The prognostic significance of miR-126 further strengthens the link between AML patient outcomes, stemness properties, and the regulatory of role of miRNA (Greaves, 2011).

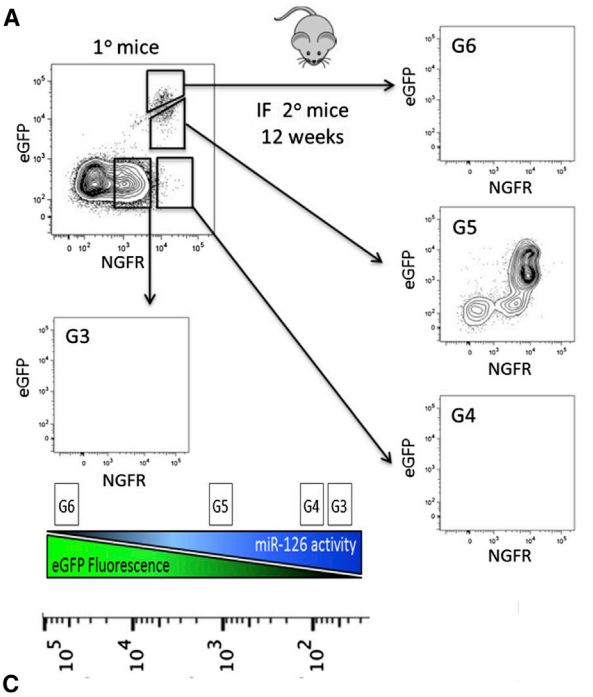
Development of a Functionally Relevant Human AML Model for Mechanistic Studies

How miR-126 functions throughout the AML hierarchy is difficult to investigate since functional studies in primary AML cells are technically challenging and hitherto no human AML cell lines recapitulate the hierarchical organization of primary cells. Therefore, we developed an indefinitely growing AML culture system (8227) from a relapse sample that is organized as a functional hierarchy (Figure 3A) (E.L., unpublished data). Expression of CD34 and CD38 is tightly linked to the functional hierarchy; $CD34^+CD38^-$ cells possess LSC activity and contain a quiescent population, by contrast $CD34^+CD38^+$ cells are enriched in clonogenic progenitors and the remaining 90% of $CD34^-CD38^+$ and $CD34^-CD38^-$ cells are terminally differentiated $CD15^+CD14^+$ blasts (Figure 3A). We show through an integrated analysis of function, phenotype, miR-126 bioactivity, and promoter methylation status on all sorted fractions that high miR-126 levels correlate with the $CD34^+CD38^-$ phenotype and LSC activity and are linked to EGFL7 expression and stem cell-specific promoter methylation patterns (Figure S3A and E.L., unpublished data). Thus, 8227 cells are a relevant model culture system for interrogating the functional effects of miR-126 activity within the context of a leukemic hierarchy.

miR-126 Expression Induces Quiescence in Primitive AML Cells

To investigate the functional importance of miR-126 within the AML developmental hierarchy, 8227 cells were transduced

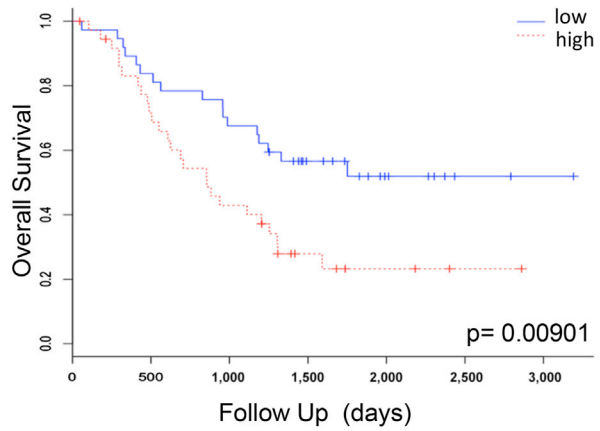
A



B

AML	Population Gate	Cell # injected per mouse	$CD45^+/NGFR^+/eGFP^+$ Engraftment	miR-126 Reporter Status
AML 1	G6	20,000	0/4 mice	Low
	G5	20,000	0/4 mice	High
	G4/G3	20,000	4/4 mice	High
AML 3	G6	6,050	0/4 mice	Low
	G5	8,500	1/4 mice	High
	G4	1,466	0/4 mice	High
	G3	100,000	0/4 mice	High
AML 7	G6	88,000	0/5 mice	Low
	G5	88,000	0/5 mice	High
	G4	50,000	0/5 mice	High
	G3	40,000	5/5 mice	High
AML 18	G6	2,100	0/4 mice	Low
	G5	18,250	3/3 mice	High
	G4	22,500	0/4 mice	High
	G3	70,500	0/4 mice	High

C



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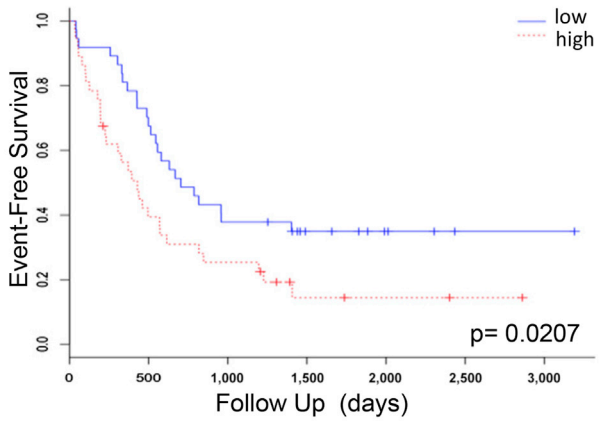

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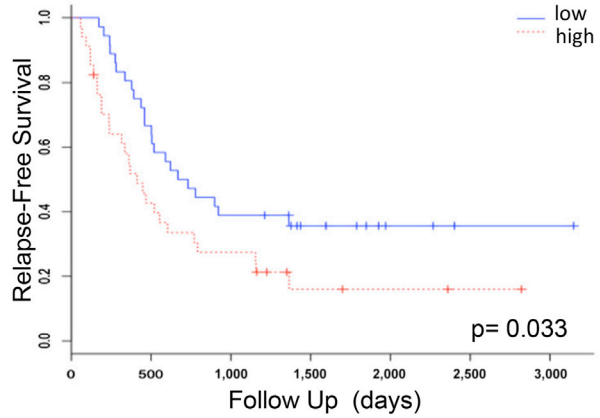

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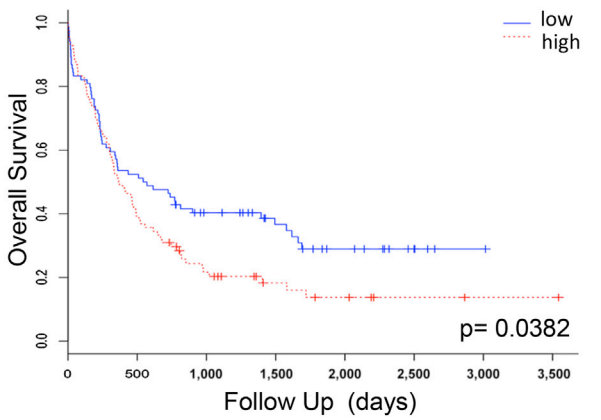

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with an mOrange (mO) lentivirus expressing miR-126 (126OE) or empty control vector (CTRL) (Figure S3B), and elevated miR-126 levels were confirmed (Figure 3B). Following in vitro propagation of transduced cells, the $mO^+CD34^+CD38^-$ (surrogate LSC) population was sorted and the proliferative, differentiation, and clonogenic capacity was evaluated over 28 days. By 7 days, primitive $CD34^+$ cells increased (Figure 3C) and differentiated $CD14^+CD15^+$ cells decreased (Figure S3C) in the 126OE group. This proportional increase in $CD34^+$ cells correlated to transient reductions in clonogenicity of day 0 bulk cultures (Figure S3D); a reduction primarily confined to $CD34^+CD38^+$ clonogenic fractions (Figure 3D). Bulk cultures of the 126OE group had significantly decreased (15%) bromodeoxyuridine (BrdU) incorporation at 3 hr ($p = 0.002$) and 16 hr ($p = 0.001$) compared with CTRL (Figure 3E). No differences in apoptosis were observed (data not shown). Cell cycle analysis of sorted 126OE populations at 7 days showed 2-fold increased proportions of quiescent (G_0) $CD34^+CD38^-$ cells and decreased $S/G_2/M$ cells (Figures 3F and S3E). By contrast, the G_0 status of 126OE $CD34^+CD38^+$ and $CD34^-$ populations remained unaffected (data not shown). Thus, 126OE maintains 8227 cells in a more primitive state by increasing the proportion of quiescent $CD34^+CD38^-$ cells, thereby decreasing the overall proliferative output and differentiation of AML blasts.

miR-126 Knockdown Provokes LSC Entry into Cycle

To determine the impact of miR-126 knockdown, 8227 cells were transduced with lentiviruses that were empty (CTRL) or expressing an miR-126 sponge (126KD) (Figure S3F) (Lechman et al., 2012). Following sorting and culture, 126KD of the $EGFP^+$ $CD34^+CD38^-$ population resulted in increased output of $CD34^+$ cells at all time points (Figure 3G), without increasing differentiation (Figure S3G). This effect was primarily localized to the $CD34^+CD38^-$ compartment (Figure 3H). Clonogenic potential within the $CD34^+CD38^-$ LSC-enriched compartment increased while no differences were observed in the $CD34^+CD38^+$ progenitor-enriched compartment (Figure 3I). 126KD increased BrdU incorporation by 20% at 3 hr ($p = 0.0024$) and 16 hr ($p = 0.0093$) (Figure 3J) without affecting apoptosis (data not shown). Upon 126KD, the proportion of cells in G_0 was decreased (30%) and $S/G_2/M$ increased (3-fold) within $EGFP^+CD34^+CD38^-$ populations (Figures 3K and S3H). 126KD of $CD34^+CD38^+$ cells trended in the same direction (CTRL G_0 16.07% versus 126KD G_0 11.54%, $p = 0.2$); $CD34^-$ and non-transduced populations were unaffected (data not shown). Within bulk 126KD cultures, the increased cell cycle and clonogenicity (Figure S3I) was primarily due to effects on $CD34^+CD38^-$ cells (Figure 3H). As LSC-enriched $CD34^+CD38^-$ cells are less clonogenic than $CD34^+CD38^+$ cells, we interpret these data as 126KD driving $CD34^+CD38^-$ cells out of their quiescent stem-like state and into a more committed population of proliferating clonogenic cells while retaining a primitive cell surface phenotype.

Enforced Expression of miR-126 Expands LSC In Vivo

To test the prediction that miR-126 maintains a primitive state by restraining entry into the cell cycle of LSC from patients, nine AML samples were transduced with 126OE and CTRL vectors and transplanted into NSG mice (Tables S2 and S3). Transduction efficiency and expression varied (Figures S4A and S4B), while leukemic engraftment was similar between CTRL and 126OE groups (Figure S4C). Although the initial transduction efficiency was ~50% lower for 126OE than CTRL in six of nine AML samples, $mOrange^+$ cells within the human $CD45^+$ graft was higher for six of nine AML samples indicating a competitive advantage for 126OE groups (Figure S4D). Analysis of primitive cell engraftment used both CD34 and CD117, as CD117 is associated with AML clinical outcome and correlated with miR-126 expression (de Leeuw et al., 2014; Schneider et al., 2015). Phenotypic primitive cells were increased in 126OE groups for seven of nine samples (Figure 4A) with concomitant reduction of differentiated cells; four of nine samples showed a significant reduction for $CD15^+$ cells (Figure 4B), and six of nine showed a trend for reduced $CD14^+$ blasts (Figure S4E). 126OE caused an increase in $CD15^+$ blasts for two samples (Figure 4B).

To evaluate 126OE on LSC function within the xenografts, serial transplantation with limiting dilution analysis was used to quantify LSC numbers. In three samples, LSC frequency increased in the 126OE group (Figures 4C and S4F). Although individual patient samples exhibited variation, overall, 126OE increased LSC self-renewal and reduced differentiation leading to LSC expansion.

miR-126 Knockdown Targets LSC In Vivo

126KD was used to determine whether reducing miR-126 would impair AML engraftment or LSC function (Figure S5A). Total levels of human $CD45^+$ (Figure S5B) or $CD45^+EGFP^+$ engraftment (Figure S5C) were unaffected in the 126KD group, although there was heterogeneity. By contrast, primitive $CD117^+$ blasts were reduced in three of seven in the 126KD group, while two of seven had increased $CD117^+$ blasts (Figure 5A); differentiated $CD15^+CD14^+$ cells were increased in four of seven samples (Figure 5B). The LSC frequency was reduced in two of three samples

Figure 2. miR-126 Bioactivity Marks the Functional LSC Compartment in Human AML

(A) Schematic describing the sorting scheme/scoring system for secondary mice. AML samples were transduced with an miR-126 reporter construct and transplanted into conditioned NSG mice for 12 weeks. Bone marrow was analyzed for engraftment using $CD45^+\Delta NGFR^+EGFP^+$ staining. Cells were sorted into four populations based on ΔNGFR (transduced cells) and EGFP expression (inverse of miR-126 bioactivity), counted, and injected into secondary mice for 8–10 weeks. When a ΔNGFR/EGFP profile is recapitulated in secondary mice, the mouse is scored as engrafted.

(B) Summary of the results of the miR-126 bio-reporter assays.

(C) Kaplan-Meier overall survival (OS) curves in the PMCC CN AML cohort (n = 74) according to the miR-126 expression level (HR, 2.23; p = 0.00901).

(D) Univariate Cox model analysis for miR-126 as prognostic for event-free survival in the PMCC cohort of CN AML patients (n = 74; p = 0.0207, log rank test, median split; HR, 1.8744; p = 0.0207, Wald test).

(E) Kaplan-Meier survival curves correlating miR-126 expression and relapse-free survival in the PMCC patient cohort. Univariate median split log rank test (HR, 1.7995; p = 0.033, Wald test).

(F) Univariate analysis for OS in the TGCA AML cohort that encompasses all levels of cytogenetic risk (n = 187) according to the miR-126 expression level (HR, 1.41; p = 0.0382). See also Figure S2.

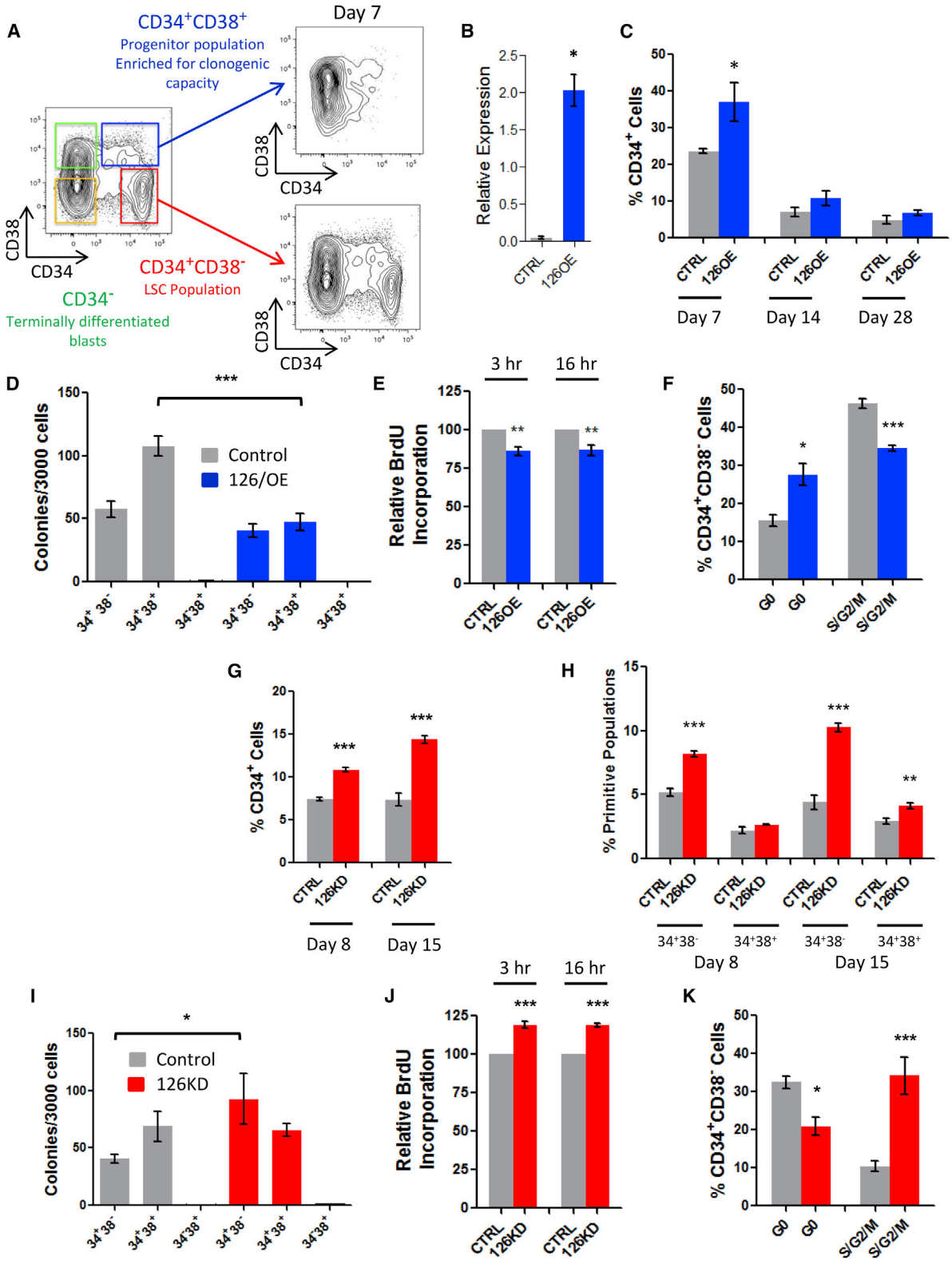

Figure 3. Enforced Expression and Knockdown of miR-126 Alters the Proliferation and Differentiation Status of Primitive 8227 AML Cells

(A) Illustration showing flow plots of CD34 and CD38 immunostained 8227 cultures. The red gated ($CD34^+CD38^-$) population is enriched in quiescent LSC and reinitiates the original hierarchy in vitro after flow sorting. The blue gated population ($CD34^+CD38^+$) is enriched in colony-forming unit (CFU) potential and

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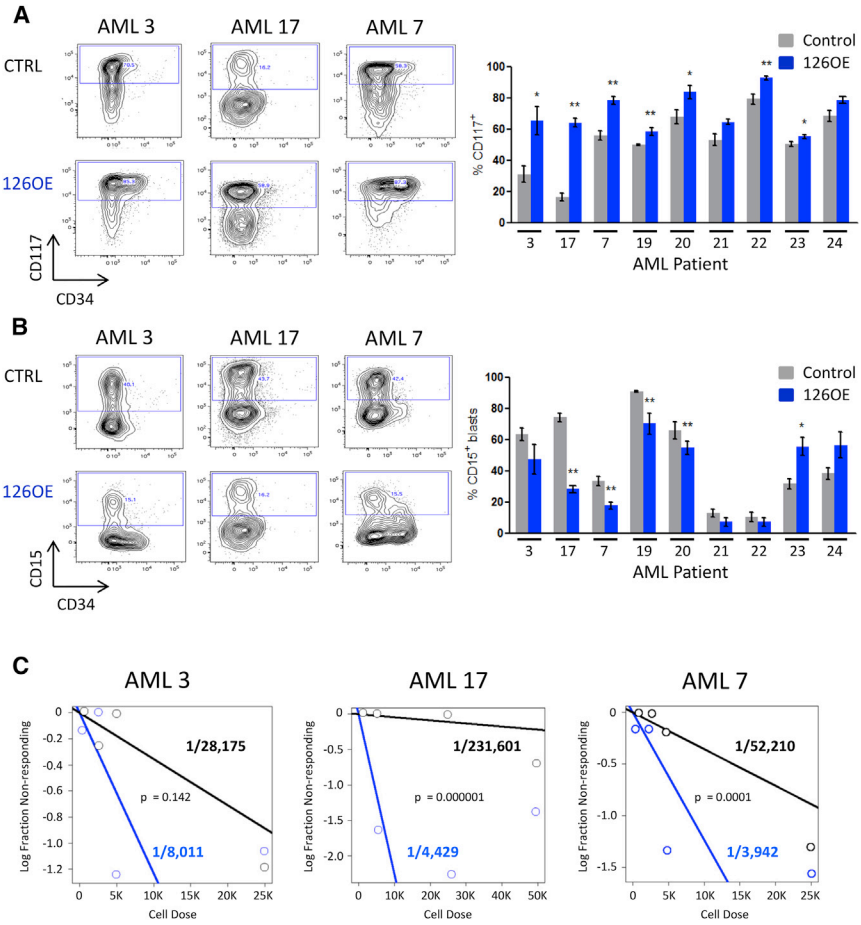

Figure 4. Enforced Expression of miR-126 Expands Primary AML LSC

(A) Representative flow plots depicting changes in CD117$^+$ and CD34$^+$ levels upon 126OE and quantification of the percentage of CD117$^+$ cells within the human CD45$^+$mO+$^+$ graft.

(B) Representative flow plots depicting changes in the percentage of CD34$^+$ and CD15$^+$ cells within the human CD45$^+$mO$^+$ graft and quantification of changes in the percentage of AML cells expressing differentiation marker CD15. Data in (A) and (B) represent means ± SEM of 4–6 mice; *p < 0.05, **p < 0.01.

(C) CD45$^+$mO$^+$ AML cells were flow sorted from primary mice and transplanted in limiting doses into secondary recipients for 8–10 weeks. Human CD45$^+$ marking of > 0.5% was considered positive for AML engraftment. Human grafts were confirmed to be CD33$^+$CD19$^-$ AML. Limiting dilution analysis was performed using ELDA software. See also Figure S4 and Table S2.

upon 126KD (Figures 5C and S5D). Together, these findings suggest that 126KD produces heterogeneous responses with LSC function and frequency reduced in a subset of AML patients.

PI3K/AKT/MTOR Is Targeted by miR-126 in Primitive AML Cells

An integrated transcriptional and proteomic approach was employed to gain mechanistic insight into miR-126 functioning. Quantitative protein mass spectrometry (MS) was performed on bulk 126OE and CTRL 8227 cells resulting in the identification and quantification of 8,848 and 4,837 proteins, respectively. In parallel, gene expression profiling was undertaken on 126KD, 126OE or CTRL CD34$^+$CD38$^-$, and CD34$^+$CD38$^+$ 8227 cells. Gene set enrichment analysis (GSEA) of the proteomics dataset identified pathways and leading edge genes directly targeted by miR-126. In post-analysis, transcriptomic datasets were correlated with proteomic-modulated pathways (Figure 6A). The most significant pathways identified centered on PI3K/AKT/MTOR signaling, a miR-126 target pathway previously validated in primitive normal human CB cells (Lechman et al., 2012). In addition, the protein MS data revealed a strong quiescence signature (Figure S6A) substantiating the in vitro cell cycle effects (Figures 3 and S3). Additional BrdU labeling studies with miR-126OE and miR-126KD confirmed these cell cycle effects in vivo (Figures S6B and S6C). The proteomic analysis was validated and confirmed by western blot of 8227 cells showing that ADAM9, PIK3R2 (p85beta), and AKT levels are reduced in 126OE groups (Figure 6B). Although AKT is not a predicted miR-126 target, the protein MS data show that all three AKT isoforms are reduced by 126OE (Table S4). In addition, many predicted and validated miR-126 targets are signaling inputs for AKT activity (Martelli et al., 2010). To activate AKT,

represents the AML progenitor compartment. Both green and orange gated CD34$^-$ compartments are devoid of LSC and CFU activity, express CD15 and CD14 differentiation markers, and represent terminally differentiated mature AML blasts.

(B) Relative expression of mature miR-126-3p in 8227 cells 7 days after transduction with lentivectors expressing miR-126 (126OE) or an empty control vector (CTRL) measured by qPCR.

(C) The proportion of CD34$^+$ cells over the time course of culture of 126OE and CTRL cells.

(D) Clonogenic potential of sorted subpopulations of 8227 cells after transduction with CTRL or 126OE vectors plated immediately post-sort.

(E) Percent BrdU incorporation into bulk cultures showing proliferation of CTRL and 126OE transduced 8227 cells over time.

(F) Ki67/Hoechst cell cycle staining of CD34$^+$CD38$^-$ LSC-enriched 8227 cells.

(G and H) Percentage of total CD34$^+$ (G) and primitive CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$ progenitor cells (H) at day 8 and day 15 post-sort in vitro in 8227 culture after sponge-mediated miR-126 knockdown.

(I) Day 0 post-sort colony-forming potential of sorted fractions of CTRL and 126KD 8227 cells.

(J) Proliferation measured by BrdU incorporation assay of CTRL or 126KD transduced 8227 cells in vitro.

(K) Cell cycle analysis of CD34$^+$CD38$^-$ 8227 cells measured by Ki67/Hoechst staining.

Data are shown as means ± SEM of three biological replicate experiments. *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S3.

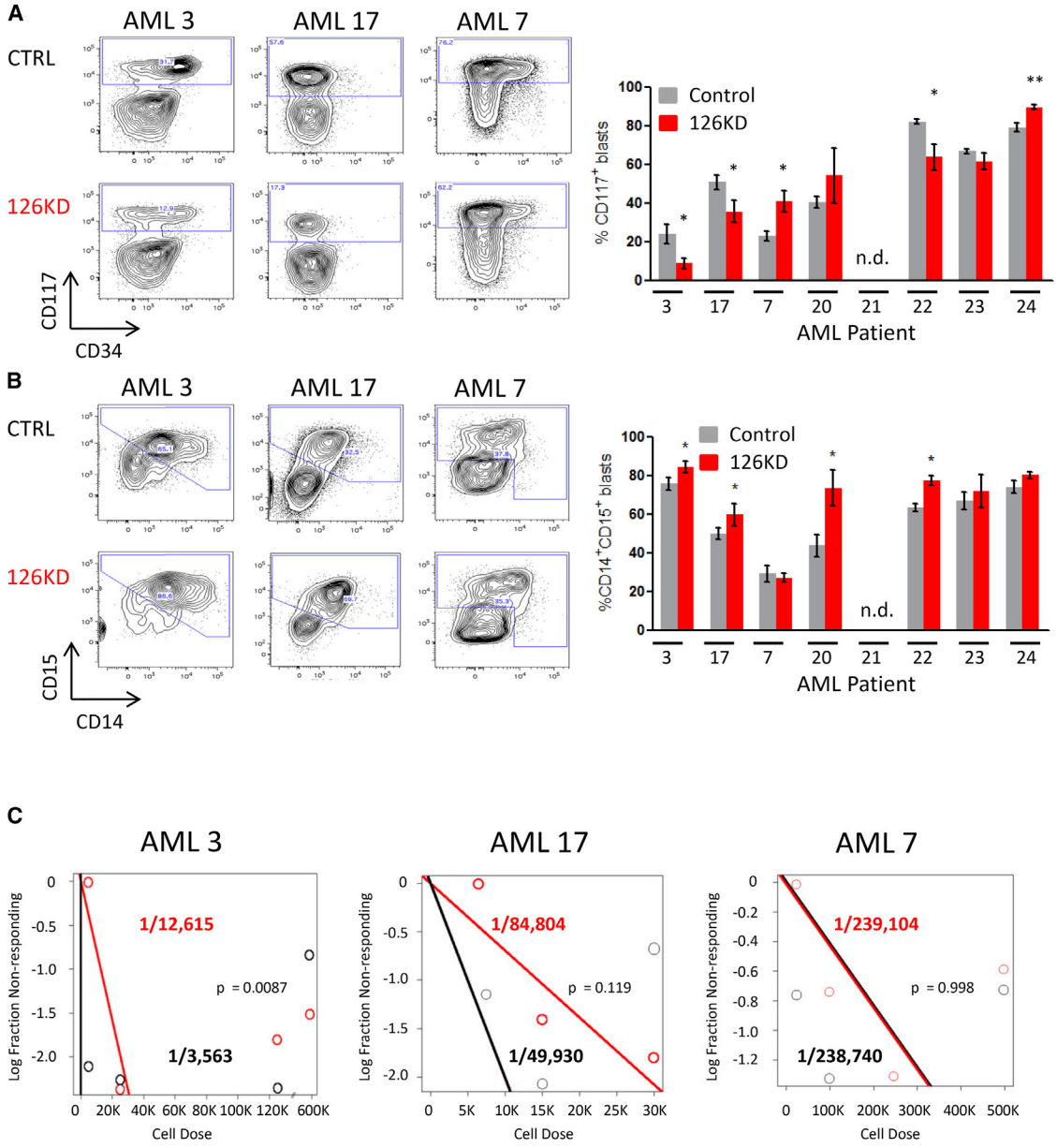

Figure 5. Diminished miR-126 Levels Reduce the Proportion of Primitive AML Cells

(A) Representative flow plots depicting changes in CD117$^+$ and CD34$^+$ levels upon 126KD and quantification of the percentage of CD117$^+$ cells within the human CD45$^+$EGFP$^+$ graft.

(B) Representative flow plots depicting changes in the percentage of CD14$^+$CD15$^+$ cells within the human CD45$^+$EGFP$^+$ graft and quantification of changes in percentage of AML cells expressing differentiation markers CD14 and CD15. Data in (A) and (B) represent means ± SEM of 4–6 mice; *$p < 0.05$, **$p < 0.01$.

(C) CD45$^+$EGFP$^+$ AML cells were flow sorted from primary mice and transplanted at limiting doses into secondary recipients for 8–10 weeks. Human CD45$^+$ marking of >0.5% was considered positive for AML engraftment. Human grafts were confirmed to be CD33$^+$CD19$^-$ AML. Limiting dilution analysis was performed using ELDA software. See also Figure S5.

PDK1 is required to phosphorylate AKT on Thr^{308} in the activation loop. We found that pPDK1 Ser^{241} is reduced with 126OE, suggesting PDK1 activity is reduced by miR-126, further dampening AKT activation (Figure 6C). MTORC2 plays a critical role in AKT Ser^{473} phosphorylation, a prerequisite for full AKT activation. Our proteomics analysis found that MAPKAP1 (Sin1) was downregulated by 126OE (Figure 6A) and since MAPKAP1 is required for MTORC2 complex formation (Yang et al., 2006), its reduction is predicted to reduce MTORC2 activity. Finally, since PTEN antagonizes the PI3K/AKT signaling pathway by dephosphorylating phosphoinositides, and no change in total PTEN levels were observed by protein MS, we checked pPTEN Ser^{380} status and found increased pPTEN Ser^{380} phosphorylation; a modification thought to stabilize PTEN and maintain its

function (Birle et al., 2002). Collectively, this integrated analysis provides strong data that miR-126 expression dampens many components of the PI3K/AKT/MTOR signaling pathway in primitive AML populations.

To characterize miR-126 targets not identified by proteomics or GSEA, all genes upregulated with 126KD and downregulated with 126OE (Figure S6D) were compared in collated lists of predicted miR-126 targets generated from four published prediction algorithms. Genes were ranked according to the level of perturbation by miR-126 (Figure S6E). Selected candidates including ADAM9, ILK, GOLPH3, CDK3, and TOM1 were confirmed as miR-126 targets using 3′ UTR luciferase reporter assays (Figure S6F) (Hamada et al., 2012; Oglesby et al., 2010).

PI3K/AKT signaling ultimately converges upon cyclins and CDK that promote RB1 phosphorylation and cell cycle entry. The uncovering of CDK3 as a potential miR-126 target was intriguing as miR-126 reduces cell cycle progression and CDK3 was previously identified as a gatekeeper of G_0-G_1 cell cycle control (Ren and Rollins, 2004). The PI3K/AKT/MTOR pathway regulates CDKN1B ($p27^{kip}$) protein stability by controlling the levels of SKP2, a component of the SCF^{SKP2} ubiquitin ligase complex (Lin et al., 2009). Both chemical inhibition of PI3K or enforced expression of PTEN induces $p27^{kip}$ upregulation in quiescent cells (Collado et al., 2000; Lu et al., 1999) and CDK3 activity is downregulated with transient $p27^{kip}$ expression (Braun et al., 1998; Hsu et al., 2000). To test the hypothesis that miR-126 modulation of PI3K/AKT/MTOR signaling influences LSC function through CDK3, functional studies were undertaken. Intracellular flow cytometry of 8227 cells showed reduced CDK3 protein levels and pRB $Ser^{807/811}$ levels upon 126OE (Figure 6D). CDK3/cyclin C phosphorylation of RB1 on $Ser^{807/811}$ is required to induce cell cycle entry from a quiescent state (G_0 exit) (Ren and Rollins, 2004) (Miyata et al., 2010) To verify that 126OE functions are dependent on CDK3 downregulation, lentiviruses expressing CDK3 or the CDK3 kinase mutant (CDK3mut) were generated (Figure S6G) (van den Heuvel and Harlow, 1993). Compared with CDK3mut, CDK3 significantly increased proliferation and clonogenicity of $CD34^+CD38^-$ and $CD34^+CD38^+$ cells (Figures 6E and 6F), and partially reversed 126OE-induced expansion of $CD34^+$ cells (Figures 6G and S6H). Collectively, these data suggest that miR-126 restricts LSC proliferation partly through targeting CDK3.

High miR-126 Bioactivity Endows LSC with Chemotherapy Resistance

To test whether the induction of LSC quiescence by 126OE is associated with chemotherapy resistance, 126OE or CTRL transduced 8227 cells were exposed to increasing concentrations of daunorubicin. 126OE increased the survival of $CD34^+$ cells after 72 hr of treatment compared with CTRL (Figure 7A), an effect not seen in non-transduced cells (Figure S7A). Treatment of primary AML samples (Table S5) with daunorubicin plus cytarabine resulted in enrichment of primitive $CD117^+$ cells (Figure S7B) and increased miR-126 levels in four of five samples (Figure 7B). Thus, primitive AML cells expressing the highest miR-126 levels are also the most resistant to anti-proliferative chemotherapy.

To determine if miR-126 expression could be linked to chemotherapy resistance in a clinical setting, biobanked samples were identified from eight AML patients who failed to achieve complete remission after induction therapy. $CD45^{dim}$ blasts were isolated from bone marrow at diagnosis (n = 8, day 0), day 14 (n = 4), and day 30 (n = 5) post-induction, and at day 30 after salvage chemotherapy (n = 3). In line with the in vitro findings, miR-126 expression was increased in six of eight samples (median, 3.4-fold; range, 0.3–9.4) after induction, and in two of three patients (including one in whom miR-126 expression was unchanged after induction) following salvage chemotherapy (median, 1.8-fold; range, 1.1–2.1) (Figures 7C and S7C). miR-126 expression was higher in relapse blasts compared with paired diagnostic samples in all four patients tested (Figures 7D and S7D). miR-126 expression in primitive $CD45^{dim}CD117^+$ cells was increased in eight of ten patients at relapse, with >100-fold enrichment in two patients (Figures 7E and S7E). Finally, enforced expression of CDK3 in 8227 cells rescued the 126OE effects by decreasing the proportion of $CD34^+$ cells resistant to daunorubicin and cytarabine (Figure 7F). Overall, these data suggest that miR-126 confers resistance to chemotherapy, likely through the induction and maintenance of cellular quiescence by the targeting and repression of the PI3K/AKT/MTOR pathway.

DISCUSSION

Our study establishes that miRNAs play a powerful role in governing the fundamental properties that define the stemness state of human LSC including quiescence, self-renewal, and chemotherapy response. miRNAs are differentially expressed within distinct cellular subsets that make up the AML hierarchy, with a restricted set expressed in an LSC-specific manner. The miRNA LSC signature was itself highly prognostic. This clinical association, together with the miR-126 functional data, establishes that miRNAs provide a layer of post-transcriptional control critical for maintaining the stemness state in AML. Although miR-126 governs the stemness and quiescence properties of both HSC and LSC, miR-126 perturbation results in divergent self-renewal outcomes. This discordance provides a novel avenue to therapeutically target LSC without attendant toxicity to HSC.

Our study provides a mechanistic link between quiescence control and the restraint of CDK3 expression by miR-126, thereby altering RB1 phosphorylation and delaying G_0 exit in human primitive AML populations. Regulation of G_0 exit kinetics is a fundamental HSC property, distinct from downstream progenitors, that is essential for maintaining HSC pool integrity (Laurenti et al., 2015; Nygren et al., 2006). CDK3 is poorly studied since all inbred mice carry a nonsense mutation in CDK3 (Ye et al., 2001). In quiescent human fibroblasts, CDK3 can complex with CCNC (cyclin C) and phosphorylate Rb1 (on residues S^{807} and S^{811}) to directly initiate the cell cycle; when CDK3 levels are reduced, a 12-hr lag in G_0 exit kinetics is induced but not a permanent block (Ren and Rollins, 2004). In murine LT-HSC, CCNC levels are highest during G_0 exit (Passegué et al., 2005) and CCNC knockdown in human HSC increased quiescence, promoted HSPC expansion, and increased repopulation capacity (Miyata et al., 2010). In leukemia, CCNC deletion highly correlates with relapse (van Delft et al., 2011). Thus, it is likely that the miR-126/CDK3 regulatory axis also governs G_0 exit kinetics in LSC, thereby providing new therapeutic opportunities for targeting quiescence control of LSC.

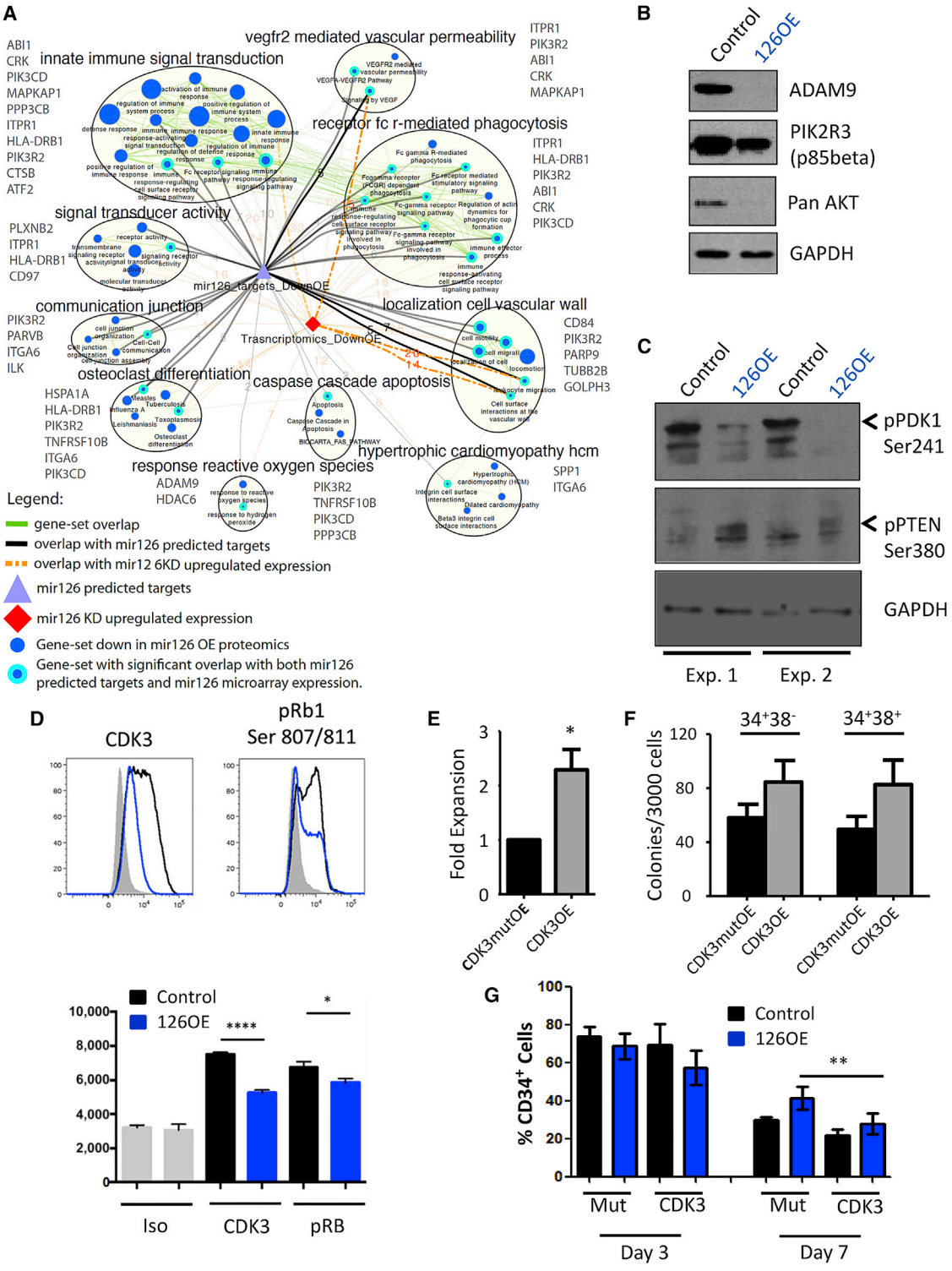

Figure 6. PI3K/AKT/MTOR Is Targeted by miR-126 in Primitive AML Cells

(A) Functional enrichment map for protein MS-based expression revealing miR-126 modulated pathways. Blue nodes (circles) represent gene sets enriched in proteins downregulated in 8227 cells overexpressing miR-126. Green line (edge) width between nodes corresponds to the number of shared proteins. Predicted miR-126 targets (purple triangle) are connected to enriched pathways by gray edges and edge width is proportional to the overlap significance (Wilcoxon proteomics $p < 0.05$ and hypergeometric test $p < 0.05$). Downregulated genes from the transcriptomics data (red diamond) are connected to enriched pathways by orange edges (Wilcoxon proteomics $p < 0.05$, Wilcoxon transcriptomic $p < 0.25$, and hypergeometric $p < 0.05$). Thickest orange and gray edges have significant Wilcoxon and Fisher's exact test $p < 0.05$. Map includes only nodes (cyan border) that have significant overlap with miR-126 predicted targets and

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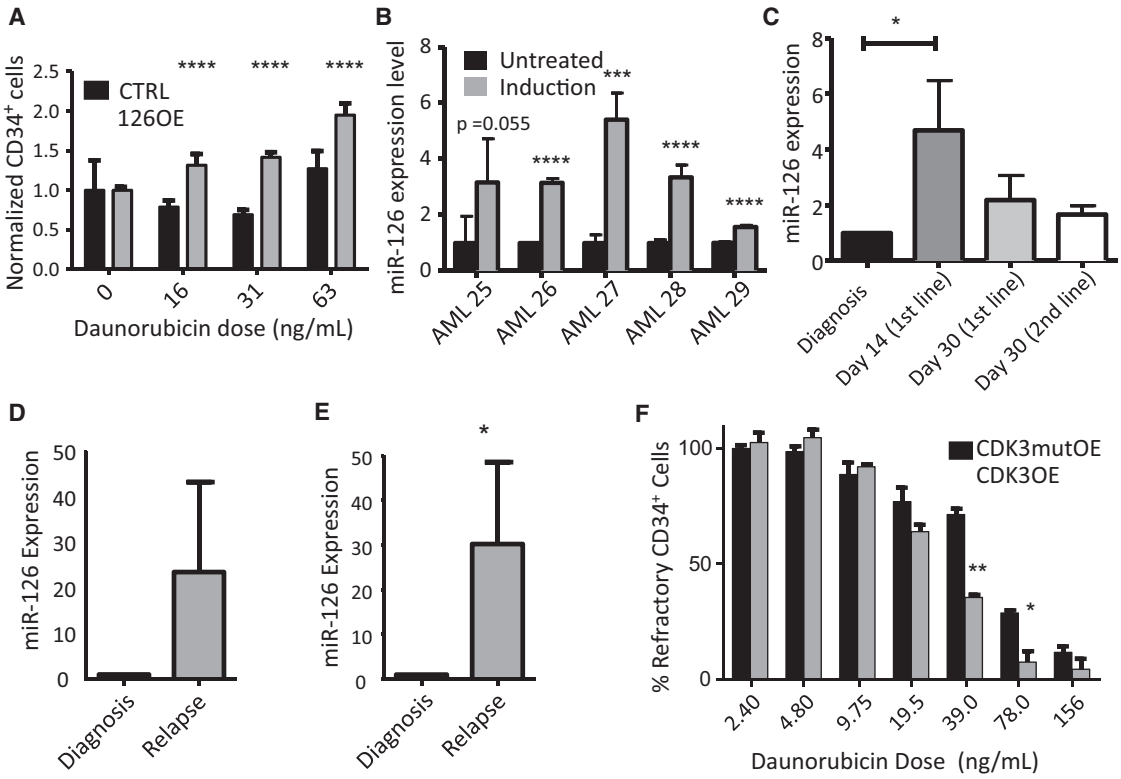

Figure 7. High miR-126 Bioactivity Endows LSC with Chemotherapy Resistance

(A) Graphical representation of percent viable CD34$^+$ 8227 cells with increasing doses of daunorubicin. CD34$^+$ cell numbers were normalized to day 0 control transduced cells. Results are shown as the mean ± SEM of three biological replicate experiments; ****p < 0.0001.

(B) Primary patient AML cells were plated onto MS5 stroma; after 24 hr cells were treated with vehicle or with daunorubicin (50 ng/ml)/AraC (500 ng/ml) for 72 hr. The miR-126 expression levels in daunorubicin/AraC-treated and control AML blasts were determined by qPCR. Results were normalized to RNU48 and are shown as the mean ± SD of four replicates; ***p < 0.001, ****p < 0.0001.

(C) qPCR was performed on CD45^{dim} sorted blasts from patient samples at diagnosis (n = 8, day 0) and at day 14 (n = 4) and day 30 (n = 5) after initiation of induction chemotherapy, as well as on day 30 after (unsuccessful) salvage chemotherapy (n = 3). Data shown are pooled from individual patients (see Figure S7C) and are shown as means ± SEM of combined individual patient samples. *p < 0.05.

(D and E) qPCR results of the relative levels of miR-126 in CD45^{dim} (D, four AML patients, see Figure S7D) and CD45^{dim}CD117$^+$ (E, ten AML patients, see Figure S7E). AML blasts in paired diagnosis and relapse patient samples shown as the mean ± SEM of all patients combined; *p < 0.05.

(F) 8227 cells transduced with mutCDK3 and CDK3 lentiviruses were plated into a 96-well plate and treated with increasing doses of daunorubicin for 48 hr. Cells were stained for CD34 and live cells were identified by viability dye exclusion by flow cytometry. Results are shown as the mean ± SEM of four biological replicates; *p < 0.05 and **p < 0.01. See also Figure S7 and Table S4.

A model derived from our proteomic and transcriptomic data (Figure 8) depicts that upstream of CDK3, miR-126 represses multiple inputs converging on PI3K/AKT/MTOR signaling in LSC, paralleling miR-126 function in HSC (Lechman et al., 2012). Preclinical evidence indicates that activated PI3K/AKT/MTOR signaling plays a role in AML (Martelli et al., 2010) despite

expression data and connected nodes belonging to same clusters (MCL cluster algorithm called from ClusterMaker2). Gene names in gray beside each cluster are the genes that are found in the specified cluster and overlap with predicted miR-126 targets repressed in 126OE.

(B) Western blot of ADAM9, PIK3R2, and AKT levels in 8227 cells transduced with miR-126OE or control lentivirus. GAPDH is the loading control.

(C) Western blot of phospho-PDK1 Ser^{241} and phospho-PTEN Ser^{280} levels in 8227 cells transduced with miR-126OE or control lentivirus. GAPDH is the loading control.

(D) Representative intracellular flow plots for the detection of CDK3 and pRB Ser$^{807/811}$. Graph below represents three independent intracellular flow experiments for each condition where the mean fluorescence intensity was compared. Mean ± SEM; *p=<0.05 and ****p=<0.0001.

(E) Graph depicting enhanced expansion of bulk 8227 cultures after enforced expression of CDK3 and mutCDK3. Fold expansion is normalized to mutCDK3 control culture day 7 after transduction. Data shown are the mean ± SEM of three replicate experiments; *p=<0.05.

(F) Graph showing clonogenic potential of primitive AML cells after enforced expression of CDK3 and mutCDK3. Colony counts are shown as the mean ± SEM of three replicate experiments.

(G) Graph depicting CDK3/OE rescue of CD34$^+$ cell expansion upon 126OE. 8227 cells were transduced with miR-126, and CD34$^+$CD38$^-$ cells were sorted and placed into culture. Cells were transduced with viral vectors expressing the mutCDK3 control vector or CDK3 vector. Flow cytometry was performed at day 3 and day 7. The percentage of CD34$^+$ cells in double-transduced cultures is shown as the mean ± SEM of three replicate experiments, where **p < 0.01. See also Figure S6 and Table S3.

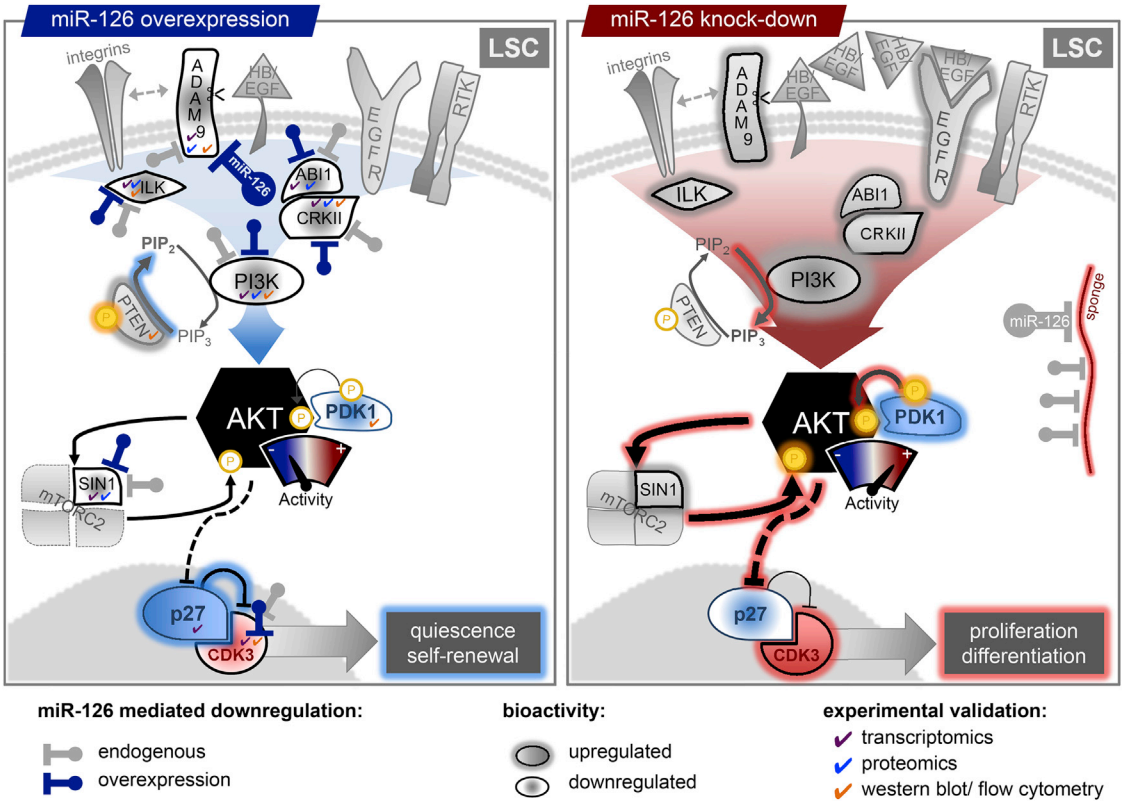

Figure 8. miR-126 Represses Multiple AKT Inputs in LSC

LSC express high endogenous levels of miR-126 compared with more differentiated AML populations. High levels of endogenous or experimental miR-126 repress the level of several proteins regulating AKT (PI3K signaling, PI3CD, PIK3R2; integrin signaling, ADAM9, ITGA6, ILK, PARVB; RTK signaling, CRK, ABI1, CD97, CD84; MTOR signaling, MAPKAP1), reducing overall AKT levels and activity. Furthermore, high levels of miR-126 reduce pPDK1 Ser^{241}, which phosphorylates AKT, and MAPKAP1, which is required for MTORC2 formation and full activation of AKT. Significantly diminished levels of AKT activity preferentially retain LSC in a quiescent state by increasing p27 levels, together with miR-126 targeted reduction of CDK3. Under high miR-126 levels, LSC that do enter the cycle are biased toward a self-renewal division. Reduction of LSC miR-126 levels through currently unspecified developmental cues (or lentiviral sponge-mediated) de-represses the expression and activity of multiple AKT signaling inputs. LSC now preferentially cycle and are biased toward differentiation divisions.

being rarely mutated and likely driven by upstream activation (Fransecky et al., 2015). Although inhibitors of AKT, MTOR, and PI3K are in clinical development, they have mostly failed for AML (Fransecky et al., 2015). While failure is attributed to feedback loops, our study provides an explanation that is embedded in the hierarchical organization of AML. PI3K/AKT/MTOR signaling is restricted to cycling leukemic progenitors; by contrast, quiescent LSC, a reservoir of leukemic relapse, have lower signaling and would then be spared following inhibitor treatment. In support of this prediction, AKT inhibition increased the fraction of G_0 breast cancer cells, linking low AKT signaling to a G_0-like state (Dey-Guha et al., 2011). In AKT knockout mice, HSC persisted in an enhanced G_0 quiescent state, while AKT activation results in HSC hyper-proliferation and exhaustion (Juntilla et al., 2010; Kharas et al., 2010). Collectively, these reports suggest that the state of AKT activity plays a key role in governing quiescence of normal HSC and our data extend this concept to leukemia by showing that this pathway is tightly controlled at multiple points by miRNAs in order to maintain the human LSC state.

Although cell cycle regulation by miR-126 is similar between HSC and LSC, the functional consequence is the opposite: reduced miR-126 levels expand HSC in vivo, but impair LSC maintenance (see the model in Figure 8). With the exception of PTEN, known regulators of self-renewal have similar functions in normal and leukemic contexts (Yilmaz and Morrison, 2008). PTEN is rarely mutated in AML, yet experimental deletion results in HSC loss and LSC expansion, supporting our data on functional HSC–LSC divergence. Concordant with our findings of low PI3K/AKT/MTOR signaling in dormant LSC, rapamycin treatment only eliminates LSC during the early phases of leukemic initiation in PTEN mouse models when LSC are proliferating, but not when leukemia is fully developed and when some LSC are predicted to re-enter quiescence (Yilmaz and Morrison, 2008).

While targeting stemness represents a promising clinical direction, finding a selective therapeutic window might be challenging due to the shared determinants of stemness between HSC and LSC, and the likelihood of causing excessive toxicity (Kreso and Dick, 2014). The distinct function of miR-126 in HSC and LSC provides an opportunity to clinically target LSC while sparing HSC. Moreover, inhibiting miR-126 might overcome LSC chemoresistance through cycle activation and increasing sensitivity to anti-proliferative drugs. Although targeting miRNA in vivo is still

inefficient (Brown and Naldini, 2009), LNA miRNA decoy technology is effective clinically in hepatitis C (Janssen et al., 2013). Alternatively, targeting the LSC-specific pathways identified by miR-126 might also be an effective strategy.

EXPERIMENTAL PROCEDURES

Patient-Derived Xenografts

NOD/Lt-scid/IL2Rγnull (NSG) mice were bred at the University Health Network/Princess Margaret Cancer Center. Animal experiments were performed in accordance with national and institutional guidelines approved by the Canadian Counsel on Animal Care and approved by the University Health Network Animal Care Committee. Mouse xenografts were performed as described previously (Lechman et al., 2012). Briefly, NSG mice were sublethally irradiated (225 cGy) 1 day prior to injection. AML patient samples were thawed and plated in X-VIVO/20% BIT (Stem Cell Technologies) supplemented with Flt3-L (50 ng/ml), IL-6 (10 ng/ml), stem cell factor (50 ng/ml), thrombopoietin (125 ng/ml), IL-3 (10 ng/ml), granulocyte colony-stimulating factor (10 ng/ml) for 18 hr (Blair et al., 1998). Cells were transduced in 24-well culture plates at a multiplicity of infection of 30 with sensor lentivectors or for enforced expression and knockdown of miR-126. Transduced AML cells (5×10^5–1×10^6) were injected with 25 μl of PBS into the right femur of each recipient mouse. After 10–12 weeks, the mice were euthanized and bone marrow cells were flushed with 2 ml of PBS, 2% fetal calf serum, and 50 μl of cells were stained for surface markers.

Patient Samples and Treatment Protocols

Between 2003 and 2010, peripheral blood and bone marrow samples were collected from subjects with AML after obtaining informed consent according to procedures approved by the Research Ethics Board of the University Health Network (REB# 01-0573-C). Mononuclear cells were isolated and stored as previously described (Eppert et al., 2011). Cytogenetics were analyzed according to the revised MRC prognostic classification system (Grimwade et al., 2010). *NPM1* and *FLT3*-ITD mutations were assessed as previously described (How et al., 2012).

The 74 patient samples used to optimize the miRNA prognostic signature (PMCC cohort) were diagnostic samples from individuals with de novo AML and normal cytogenetics. Although patients were not treated uniformly, all initially received induction chemotherapy followed by two cycles of consolidation in those who achieved complete remission (CR). First-line induction regimens included 3 + 7 (n = 69), NOVE-HIDAC (n = 1), and four patients were enrolled in clinical trials employing a 3 + 7 backbone with gemtuzumab ozogamicin (n = 2) or tipifarnib (n = 2). Treatment protocols were as previously described (Brandwein et al., 2008; Brandwein et al., 2009; How et al., 2012; Petersdorf et al., 2013). Allogeneic stem cell transplant (allo-SCT) was performed for high-risk patients in CR1 (n = 7), as well as for patients who achieved a second remission after relapse (n = 12) if they had an available donor, were younger than 70 years, lacked significant comorbidities, and had good performance status. Bio-informatic and clinical information for a second cohort of 187 de novo AML patients was obtained from TCGA and has been previously described (Cancer Genome Atlas Research Network, 2013).

See Supplemental Experimental Procedures for additional methods.

ACCESSION NUMBERS

miRNA array, Illumina array, Nanostring data have been submitted to Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) with the following series accession numbers: miRNA, GEO: GSE55917; Illumina, GEO: GSE55814; and Nanostring, GEO: GSE55770. The MS data have been deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PRIDE: PXD001994.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.ccell.2015.12.011.

AUTHOR CONTRIBUTIONS

Project Conceptualization, E.R.L, B.G., J.E.D., L.N, K.E., M.M., and J.C.Y.W.; Methodology, E.R.L. and K.E.; Investigation, E.R.L., E.M.S., P.V.G., N.T., S.M.D., A.T.G., G.K., J.E., A.M., W.C.C., K.G.H., K.E., R.M., B.L.E., J.L., and S.N.; Resources, M.M.; Data Curation, J.K.; Writing-Review and Editing, E.R.L. J.C.Y.W., and J.E.D.; Supervision, J.E.D., L.N., G.D.B., P.Z., and T.G.; Formal Analysis, S.W.K.N., J.K., B.N., R.I., V.V.; Visualization, K.K.; Funding Acquisition, T.G., J.E.D., and L.N.

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Supplemental Information

miR-126 Regulates Distinct Self-Renewal Outcomes in Normal and Malignant Hematopoietic Stem Cells

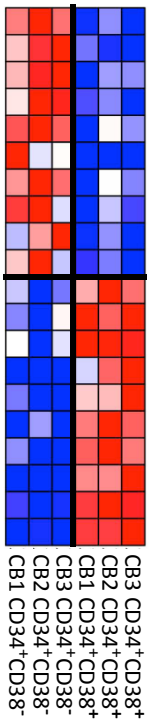
Eric R. Lechman, Bernhard Gentner, Stanley W.K. Ng, Erwin M. Schoof, Peter van Galen, James A. Kennedy, Silvia Nucera, Fabio Ciceri, Kerstin B. Kaufmann, Naoya Takayama, Stephanie M. Dobson, Aaron Trotman-Grant, Gabriela Krivdova, Janneke Elzinga, Amanda Mitchell, Björn Nilsson, Karin G. Hermans, Kolja Eppert, Rene Marke, Ruth Isserlin, Veronique Voisin, Gary D. Bader, Peter W. Zandstra, Todd R. Golub, Benjamin L. Ebert, Jun Lu, Mark Minden, Jean C.Y. Wang, Luigi Naldini, and John E. Dick

SUPPLEMENTAL DATA

A

AML	Relapse or Diagnosis	FAB	Age	Sex	Karyotype	Engraftment of AML Subpopulations			
						$CD34^+$ $CD38^-$	$CD34^+$ $CD38^-$	$CD34^-$ $CD38^+$	$CD34^-$ $CD38^-$
1	Relapse	M2	48	F	46, t(2;21)(p21;q22)[4]/46,9(1;21)(q22;q22)	+	+	-	-
2	Diag	M5a	58	F	46, XX	+	+	-	-
3	Diag	unclass	52	F	47, XX, +8	+	+	+	+
4	Diag	unclass	62	M	46, XY	+	+	-	+
5	Diag	M5a	39	F	47, XX,+8	+	+	-	-
6	Diag	unclass	80	F	46, XX	+	+	-	-
7	Diag	M5	48	M	no data	+	NT	NT	+
8	Diag	M1	72	F	46, XX	+	-	-	-
9	Diag	M2	47	F	46,t(2:21)[4]/t(6:21)[2]/t(15:21)[2]	+	NT	NT	NT
10	Diag	M2	62	F	47,XX, +13	+	NT	NT	NT
11	Diag	M1	45	F	46, XX	+	+	-	-
12	Diag	M4eo	39	M	46,inv(16)(p13;q22)	+	-	NT	-
13	Diag	M5a	75	M	46, XX	-	-	-	-
14	Diag	M4	23	M	46, XY	NT	-	-	-
15	Diag	M5a	40	M	46, XY	+	+	-	-
16	Diag	M5b	80	M	no data	NT	-	-	-

B



Probe	q value
hsa-miR-10a	0.34
hsa-miR-23b	0.39
hsa-miR-125a-5p	0.51
hsa-miR-342-3p	0.58
hsa-miR-10b hairpin	0.60
hsa-miR-326	0.60
hsa-miR-92b	0.74
hsa-miR-10b	0.76
hsa-miR-92a	0.76
hsa-miR-23a	0.76
hsa-miR-15b	0.59
hsa-miR-let-7d	0.58
hsa-miR-20b	0.58
hsa-miR-128	0.58
hsa-miR-98	0.58
hsa-miR-130b	0.34
hsa-miR-766	0.34
hsa-miR-486-5p	0.13
hsa-miR-20a	0.01
hsa-miR-181c	0.01

C

Population	Probe	q value
$CD34^+/CD38^-$		
	hsa-mmu-let-7b	0.0000
	hsa-mmu-let-7f	0.0138
	hsa-mmu-let-7a	0.0155
	hsa-mmu-miR-326	0.0371
$CD34^+$		
	hsa-mmu-let-7b	0.0000
	hsa-mmu-let-7a	0.0049
	hsa-mmu-let-7c	0.0089
	hsa-mmu-let-7f	0.0181
	hsa-miR-486-5p	0.0854
	hsa-mmu-miR-451	0.0854
	hsa-mmu-542-3p	0.0089
	hsa-miR-299-5p	0.0087
	hsa-mmu-miR-363	0.0013

Population	Probe	q value
BULK		
	hsa-mmu-let-7b	0.0000
	hsa-mmu-miR-425	0.0009
	hsa-mmu-let-7a	0.0061
	hsa-mmu-let-7c	0.0579
	hsa-mmu-miR-221	0.0579
	hsa-miR-520a-5p	0.0986
	hsa-miR-329	0.0885
	hsa-miR-193a-3p	0.0579
	hsa-miR-598	0.0566
	hsa-miR-525-3p	0.0490
	hsa-mmu-miR-26a	0.0325
	hsa-mmu-miR-542-3p	0.0064
	hsa-mmu-miR-186	0.0036
	hsa-miR-202	0.0026
	hsa-miR-299-5p	0.0013
	hsa-mmu-miR-363	0.0006

Figure S1, related to Figure 1. Generation an HSC-enriched miRNA signature and bioinformatic comparison of miRNA enriched in AML and CB fractions.

(A) Table detailing the clinical attributes of 16 AML patient samples used for the generation of a miRNA enriched LSC signature. A (+) denotes a sorted AML fraction the contained leukemia-initiating capacity in vivo, while (-) signifies no such activity was present after transplantation. (NT) indicates fractions that were not tested.

(B) Heat map depicting the top 10 miRNA candidates enriched within the human $CD34^{+}CD38^{-}$ HSC compartment and those enriched within the $CD34^{+}CD38^{+}$ committed hematopoietic progenitor population.

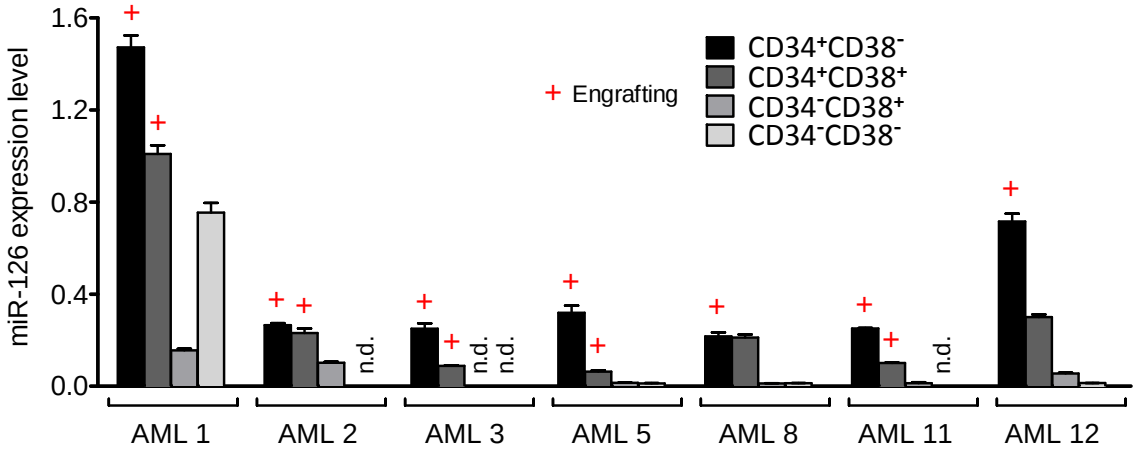
(C) Table of statistically significant miRNA probes enriched in AML (red type) or human lin^{-} CB (blue type) after informatic comparison of normal CB and malignant $CD34^{+}CD38^{-}$, $CD34^{+}$ or bulk cellular populations.

Table S1, related to Figure 1. Clinical and Molecular Characteristics of Patients Analyzed via Nanostring (PMCC Cohort)

	n=74
Male sex [n (%)]	36 (49%)
Age at AML diagnosis [years]	
median (range)	51.4 (23.2-75.9)
WBC count at diagnosis ($x10^9/L$)	
median (range)	18.2 (0.3-207)
% Blasts in PB at diagnosis	
median (range)	57 (0-98)
% Blasts in BM at diagnosis	
median (range)	70 (20-96)
Sample Material	
Bone Marrow [n (%)]	57 (77%)
Peripheral Blood [n (%)]	17 (23%)
Molecular Data [n (%)]	
mutated NPM1 (n=67)	36 (54%)
FLT3-ITD (n=67)	0
Treatment Approach	
Induction	74 (100%)
Allogeneic Transplant	19 (26%)
in CR1	7 (37%)
in CR2	12 (63%)

All samples were from diagnosis and had normal cytogenetics

A



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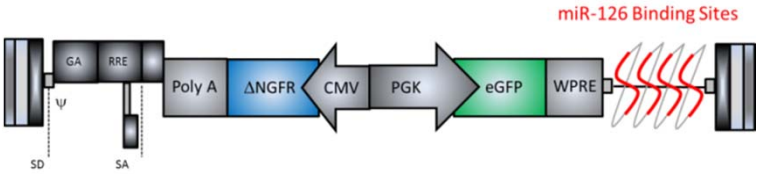
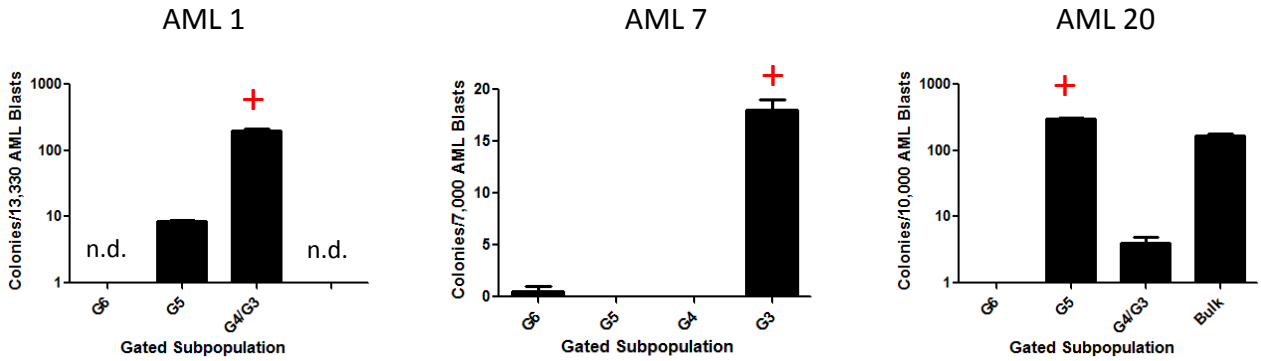


C

AML Sample	Population Gate	Fold miR-126 Enrichment from bulk	Fold miR-126 Enrichment from eGFP^{high}
AML 3	G6	0.08	1
	G5	3.2	43.1
	G4/G3	0.28	3.7
AML 7	G6	0.08	1
	G5	0.27	3.39
	G4/G3	3.39	42.3
AML 18	G6	0.02	1
	G5	1.16	45.4
	G4/G3	0.35	16

D



E

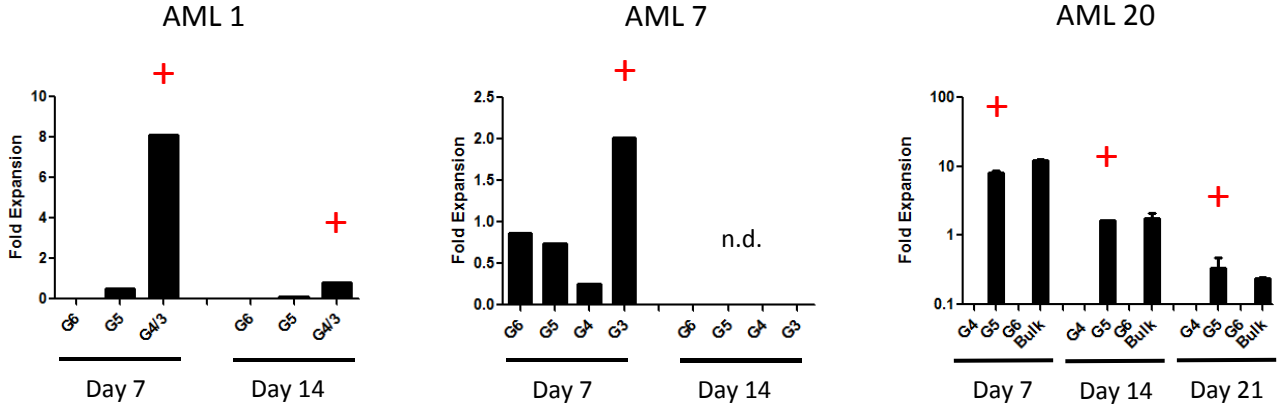


Figure S2, related to Figure 2. AML leukemia initiating capacity co-segregates with proliferation and clonogenic capacity of AML blasts.

(A) Quantitative PCR (qPCR) for mature miR-126 levels in flow sorted AML sub-fractions (see scheme depicted in **Figure 1A**). Data was normalized to RNU48 control levels. Results are shown as mean ± SEM of n=3 technical replicates.

(B) Schematic of the bi-directional miR-126 biosensor vector. The vector expresses truncated NGFR in one direction (which marks all transduced cells) and eGFP in the other direction. miR-126 binding sites are cloned downstream of eGFP. The more miR-126 a cell expresses, the less eGFP protein that is translated, leading to lower levels of eGFP fluorescence.

(C) Table depicting the normalized levels of mature miR-126-3p in biosensor sorted AML populations and measured by qPCR. In red text, the table shows the gated population most enriched in miR-126-3p levels compared to bulk AML or compared to $eGFP^{high}$ fractions (lowest miR-126 activity).

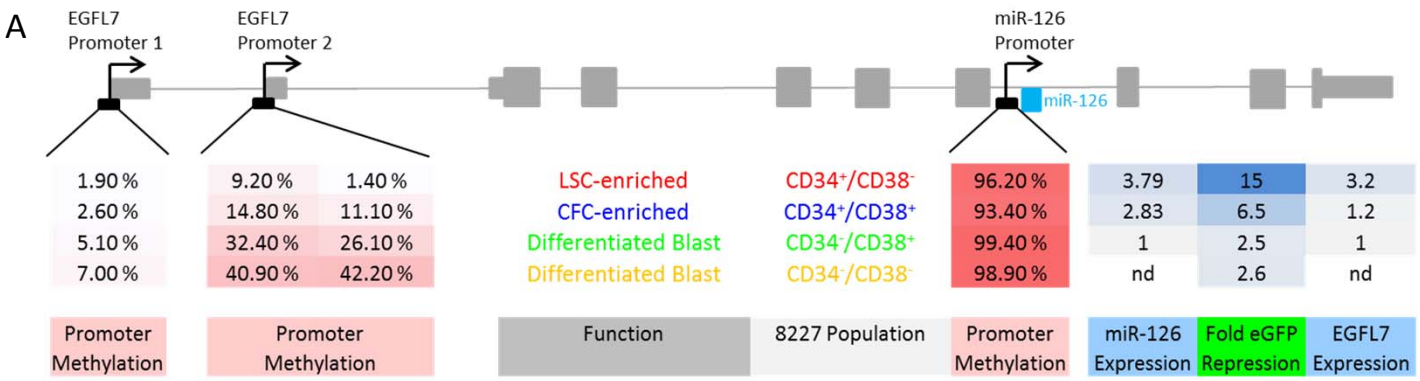
(D) Bar graphs representing the colony forming ability of flow sorted populations of miR-126 biosensor vector transduced primary AML patient samples after recovery from NSG mice. The red crosses indicate the gated populations that were able to recapitulate the AML ΔNGFR/eGFP hierarchy in vivo. Data represents mean ± SEM of n= 2 technical replicate experiments.

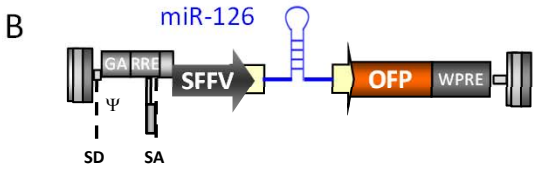
(E) Bar graphs showing proliferation capacity of flow sorted populations of miR-126 biosensor vector transduced primary AML patient samples after recovery from NSG mice. The red crosses indicate the gated populations that were able to recapitulate the AML ΔNGFR/eGFP hierarchy in vivo. Data represents mean ± SEM of n= 2 technical replicate experiments.

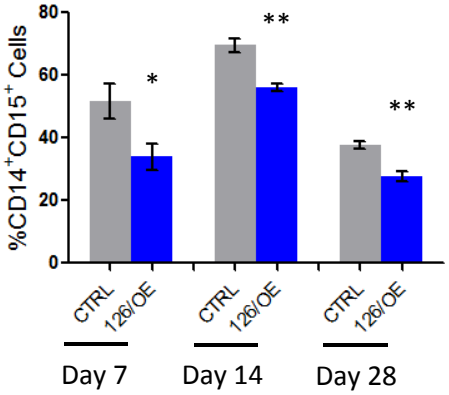
Table S2, related to Figure 2. AML Patient Samples Used for miR-126 Biosensor Lentivector in vivo Studies.

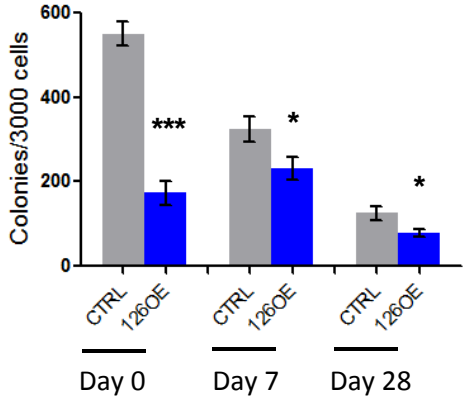
AML	Sample Type	FAB	Age	Sex	Karyotype	Engraftment of AML Subpopulations			
						$CD34^+$ $CD38^-$	$CD34^+$ $CD38^+$	$CD34^-$ $CD38^+$	$CD34^-$ $CD38^-$
1	Relapse	Unclass	48	F	46,XX,t(2;21)(p21;q22)[4]/46,XX,9(1;21)(q22;q22)	+	+	-	-
3	Diagnosis	Unclass	52	F	47,XX,+8	+	+	+	+
7	Diagnosis	Unclass	47.9	M	n.d.	+	-	-	+
18	Diagnosis	M4	34.8	M	45,XY, inv(3)(q21q26.2) -7[20]	NT	NT	NT	NT

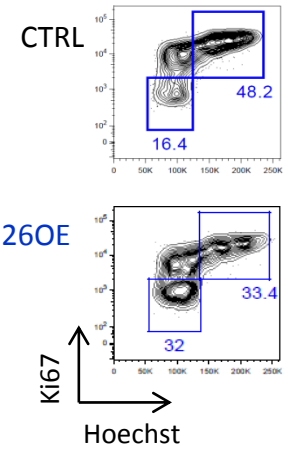
(+) symbols at right represent CD34/CD38 fractions that engraft immuno-deficient mice. (-) symbols represent fractions that were functionally evaluated and do not engraft immuno-deficient mice. (NT) symbols represent fractions that were not evaluated for LSC activity.

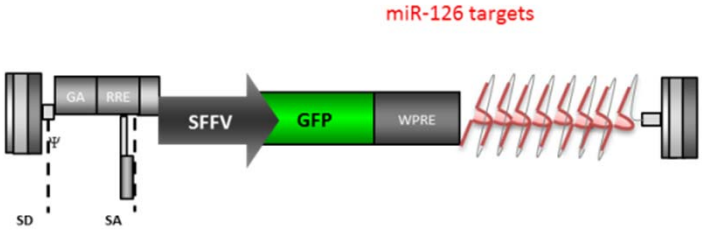

A
EGFL7 Promoter 1
EGFL7 Promoter 2
miR-126 Promoter
miR-126
1.90 %
2.60 %
5.10 %
7.00 %
9.20 %
14.80 %
32.40 %
40.90 %
1.40 %
11.10 %
26.10 %
42.20 %
LSC-enriched
CFC-enriched
Differentiated Blast
Differentiated Blast
CD34+/CD38-
CD34+/CD38+
CD34-/CD38+
CD34-/CD38-
96.20 %
93.40 %
99.40 %
98.90 %
3.79
2.83
1
nd
15
6.5
2.5
2.6
3.2
1.2
1
nd
Promoter Methylation
Promoter Methylation
Function
8227 Population
Promoter Methylation
miR-126 Expression
Fold eGFP Repression
EGFL7 Expression

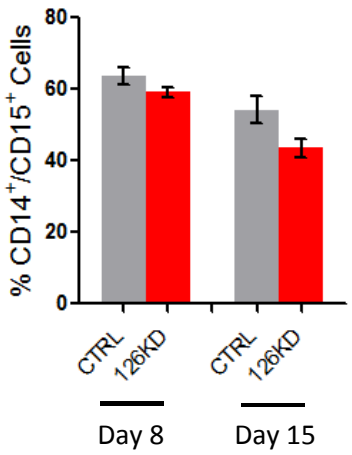

B
miR-126
GA
RRE
Ψ
SFFV
OFP
WPRE
SD
SA

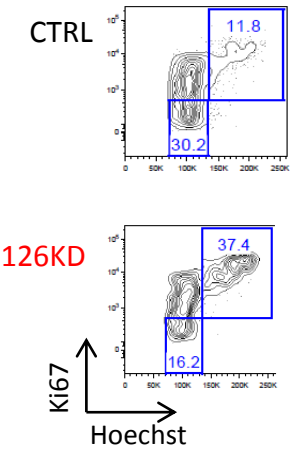

C
%CD14+CD15+ Cells
80
60
40
20
0
*
**
**
CTRL
126/OE
Day 7
Day 14
Day 28


D
Colonies/3000 cells
600
400
200
0
***
*
*
CTRL
126OE
Day 0
Day 7
Day 28


E
CTRL
48.2
16.4
126OE
33.4
32
Ki67
Hoechst


miR-126 targets
F
GA
RRE
Ψ
SFFV
GFP
WPRE
SD
SA


G
% CD14+/CD15+ Cells
80
60
40
20
0
CTRL
126KD
Day 8
Day 15


H
CTRL
11.8
30.2
126KD
37.4
16.2
Ki67
Hoechst

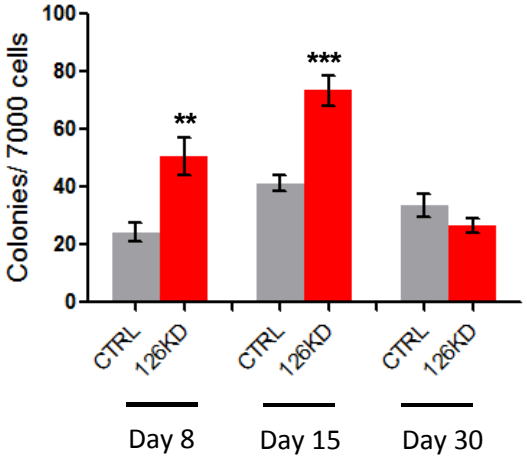

I
Colonies/ 7000 cells
100
80
60
40
20
0
**
***
CTRL
126KD
Day 8
Day 15
Day 30

Figure S3, related to Figure 3. Enforced expression and knockdown of miR-126 alters proliferation and differentiation status of primitive AML cells in vitro.

(A) Schematic of the EGFL7/miR-126 locus. Promoter methylation was determined by CD34/CD38 sorting of 8227 subpopulations. Methylated DNA immunoprecipitation (MeDIP) combined with tiling array hybridization. High methylation of the internal miR-126 promoter suggests that miR-126 expression is regulated from the EGFL7 promotor 2 site, which is progressively methylated with increasing differentiation of the 8227 cells. This differentiation is correlated with changes in cellular function as measured by in vitro culture initiation, in vivo leukemia initiation, colony forming capacity, and proliferation. Furthermore, miR-126 expression by array (normalized log2 transformed levels) and EGFL7 expression levels from array (normalized log2 transformed levels) are highest in the LSC enriched fraction and are progressively reduced with increasing differentiation. miR-126 bioactivity, as measured by the miR-126 biosensor vector, is highest in the LSC fraction and decreases with differentiation.

(B) Schematic representation of the lentiviral construct for enforced expression of miR-126. The human miR-126 hairpin is driven off of the SFFV promotor.

(C) 8227 leukemia cells were transduced with lentivectors expressing miR-126 (126OE) or an empty control vector (CTRL). Transduced cells were flow sorted for mOrange (mO^+) expression and plated in liquid culture conditions for 28 days with weekly passage. Graphical representation of the proportion of $CD14^+CD15^+$ in 8227 CTRL transduced cells or 126OE cells on days 7, 14 and 28 in post-sort cultures. Data represents mean $\pm$ SEM of n=3 replicate experiments where * $p < 0.05$, ** $p < 0.01$.

(D) Colony forming potential of CTRL and 126OE 8227 cells measured by methylcellulose CFC assay and evaluated a day 0, 7, and 28 of culture. Data represents mean $\pm$ SEM of n=2 technical replicate experiments where * $p < 0.05$, *** $p < 0.001$.

(E) Representative flow plots for cell cycle analysis with Hoechst and Ki67 cell staining of CTRL and 126/OE 8227 AML cells.

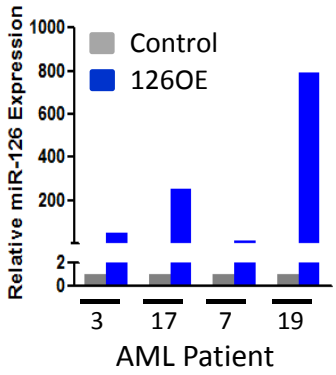
(F) Schematic representing the lentiviral sponge construct for knockdown of endogenous miR-126 activity. Eight imperfect miR-126 binding sites are cloned downstream of eGFP and driven off of an SFFV promotor.

(G) 8227 leukemia cells were transduced with lentivectors expressing miR-126 sponge (126KD) or an empty control vector (CTRL). Transduced cells were flow sorted for enhanced Green Fluorescent Protein ($eGFP^+$) expression and plated in liquid culture conditions for 30 days with weekly passage. Graph showing the proportion of $CD14^+CD15^+$ in 8227 CTRL transduced cells or 126KD cells at days 7 and 15 post-sort. Data represents mean $\pm$ SEM of n= 3 replicate experiments.

(H) Representative flow plots for cell cycle analysis with Hoechst and Ki67 cell staining of CTRL and 126KD 8227 AML cells.

(I) Graph showing colony forming potential of bulk miR-126KD 8227 cultures days 7, 15 and 30 after culture initiation. Data represents mean $\pm$ SEM of n= 3 replicate experiments where ** $p < 0.01$, *** $p < 0.001$.

A



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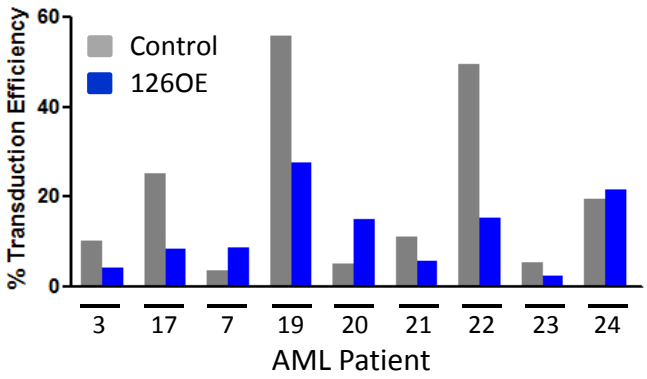

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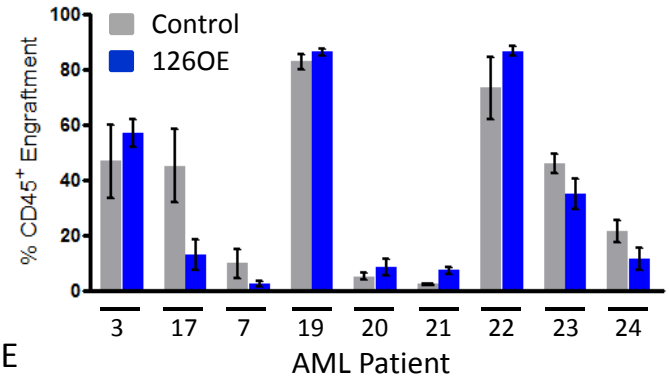

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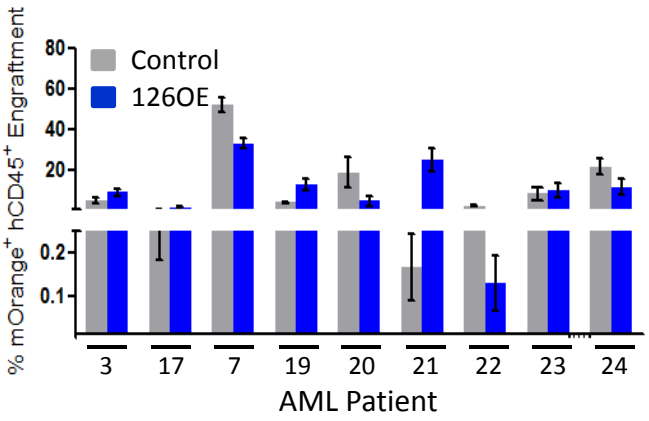

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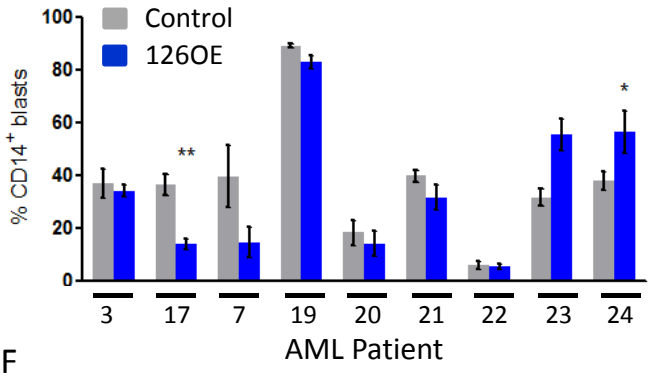


F

2° AML 3	# Cells Injected	% Mice Engrafted
CTRL	25,000	100% (2/2)
	5,000	0% (0/4)
	2,500	0% (0/5)
	500	0% (0/5)
126OE	25,000	100% (2/2)
	5,000	75% (3/4)
	2,500	0% (0/5)
	500	0% (0/5)

AML 17	# Cells Injected	% Mice Engrafted
CTRL	50,000	50% (1/2)
	25,000	0% (0/5)
	5,000	0% (0/5)
	1,000	0% (0/5)
126OE	50,000	100% (2/2)
	25,000	100% (5/5)
	5,000	80% (4/5)
	1,000	0% (0/5)

AML 7	# Cells Injected	% Mice Engrafted
CTRL	100,000	100% (2/2)
	25,000	20% (1/5)
	5,000	17% (1/6)
126OE	100,000	100% (4/4)
	25,000	100% (5/5)
	5,000	71% (5/7)

Group	Lower	Estimate	Upper
CTRL	103673	28175	7658
126OE	20845	8111	3156

Group	Lower	Estimate	Upper
CTRL	1587342	231601	33792
126OE	11021	4429	1780

Group	Lower	Estimate	Upper
CTRL	145005	52210	18799
126OE	9676	3942	1606

Figure S4, related to Figure 4. Increased miR-126 levels expand human AML LSC numbers in vivo.

(A) Relative levels of miR-126-3p expression for a subset of CTRL and 126/OE lentivirus transduced AML patient samples after 12 weeks in primary mice.

(B) Nine human AML samples were thawed, transduced overnight with lentiviruses expressing miR-126 (126/OE) or an empty vector (CTRL) and transplanted into NSG mice. After 12 weeks, mice were euthanized and bone marrow recovered for analysis. Percent AML cells transduced in 9 patient samples after overnight exposure to CTRL and 126/OE lentivirus (mOrange positivity) measured by flow cytometry 3 days post-virus transduction.

(C) Bar graph depicting changes in $hCD45^+$ levels within the bone marrow of transplanted mice after enforced expression of miR-126. Data shown represent the mean $\pm$ SEM of 4-6 mice

(D) Graphical representation of the proportion of mO^+hCD45^+cells in the bone marrow of CTRL and 126OE AML samples after 12 weeks. Data shown represent the mean $\pm$ SEM of 4-6 mice

(E) Graph of the proportion of $CD45^+mO^+CD14^+$ cells in the bone marrow of CTRL and 126OE AML samples after 12 weeks. Data shown represent the mean $\pm$ SEM of 4-6 mice where * $p < 0.05$, ** $p < 0.01$.

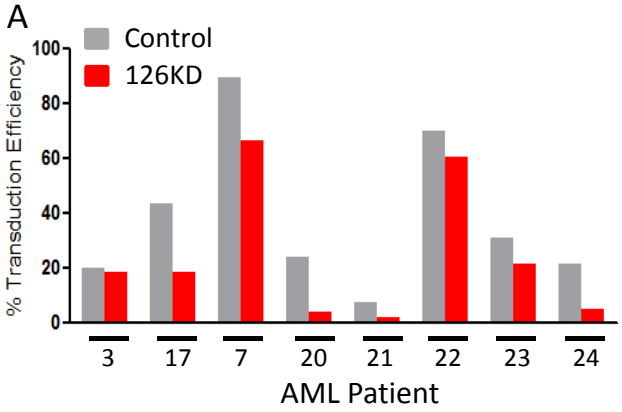
(F) Transduced human $CD45^+mO^+$ AML cells were flow sorted from primary mice and transplanted into secondary recipients for 8-10 weeks at limiting doses. Human $CD45^+$ marking > 0.5% was considered positive for AML engraftment. Human grafts were confirmed to be $CD33^+CD19^-$ AML. Tables depict transplanted cell numbers, engrafted mice percentages and numbers per CTRL and 126OE group for each AML patient sample.

Table S3, related to Figure 4. AML Patient Samples Used for in vivo Functional Evaluation of miR-126.

AML	Sample Type	FAB	Age	Sex	Karyotype	Engraftment of AML Subpopulations			
						$CD34^+$ $CD38^-$	$CD34^+$ $CD38^+$	$CD34^-$ $CD38^+$	$CD34^-$ $CD38^-$
17	Diag	Unclass	42.7	M	46, XY	+	+	+	+
19	Diag	Unclass	37.5	F	46,XX[18]: NPM1c+, FLT3-ITD+	+	+	-	+
20	Diag	M5a	60	M	45,X,-Y,t(11:19)(q23;p13.1)[20]	+	-	+	+
21	Diag	Unclass	31.5	F	46,XX,t(9;11)(p22;q23){[0]	NT	NT	NT	NT
22	Refractory	Unclass	36.7	M	46,XY,t(1;3)(q32;q26~27),del(20)(q13.1)[11]	NT	NT	NT	NT
23	Diag	M4	33.4	M	46,XY[20]; NPM1c+, FLT3-ITD+	NT	NT	NT	NT
24	Refractory	Unclass	70.7	F	46,XX,t(1;14)(q21;q11.2)[20]	NT	NT	NT	NT

(+) Symbols at right represent CD34/CD38 fractions that engraft immuno-deficient mice. (-) symbols represent fractions that were functionally evaluated by transplantation, but do not engraft immuno-deficient mice. (NT) symbols represent fractions that were not evaluated for LSC activity.

A



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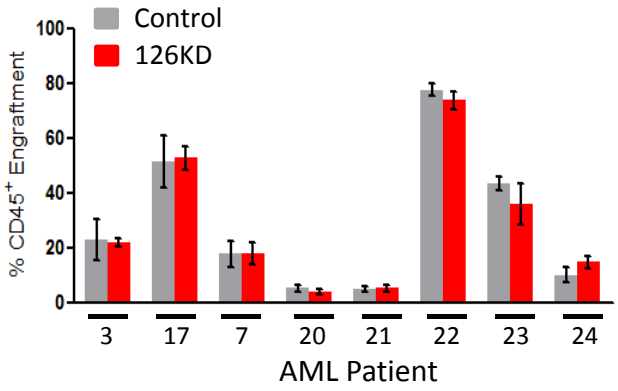

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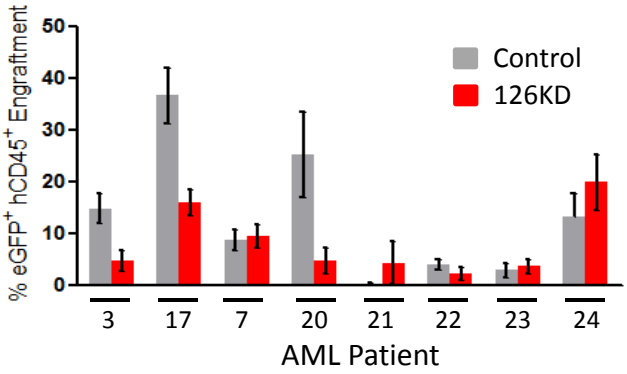

D

2°

AML 3	# Cells Injected	% Mice Engrafted
CTRL	625,000	100% (1/1)
	125,000	100% (5/5)
	25,000	100% (5/5)
	5,000	100% (4/4)
126KD	625,000	100% (2/2)
	125,000	100% (3/3)
	25,000	100% (5/5)
	5,000	0% (0/4)

Group	Lower	Estimate	Upper
CTRL	11524	3563	1102
126KD	31959	12615	4980

~3.47 Fold Decrease
In Frequency
p = 0.0087

AML 17	# Cells Injected	% Mice Engrafted
CTRL	300,000	100% (1/1)
	150,000	100% (4/4)
	75,000	100% (2/3)
126KD	300,000	100% (3/3)
	150,000	75% (3/4)
	75,000	0% (0/3)

Group	Lower	Estimate	Upper
CTRL	134514	49930	18533
126KD	197916	84804	36337

~1.7 Fold Decrease
In Frequency
p = 0.119

AML 7	# Cells Injected	% Mice Engrafted
CTRL	500,000	50% (2/1)
	250,000	100% (2/2)
	100,000	50% (2/4)
	25,000	0% (0/1)
126KD	500,000	50% (1/2)
	250,000	0% (0/1)
	100,000	75% (3/4)
	25,000	50% (1/2)

Group	Lower	Estimate	Upper
CTRL	646891	238740	88109
126KD	695315	239104	82223

No Fold Change
In Frequency
p = 0.998

Figure S5, related to Figure 5. Diminished miR-126 levels reduce the proportion of primitive AML cells

(A) Eight human AML samples were thawed, transduced overnight with lentiviruses expressing a miR-126 sponge (126KD) or an empty vector (CTRL) and transplanted into NSG mice. After 12 weeks, mice were euthanized and bone marrow recovered for analysis. Percent AML cells transduced in 8 patient samples after overnight exposure to CTRL and 126KD lentivirus (eGFP positivity) measured by flow cytometry 3 days post-virus transduction.

(B) Bar graph depicting changes in $hCD45^+$ levels within the bone marrow of transplanted mice with reduced expression of miR-126. Data shown represent the mean $\pm$ SEM of 4-6 mice.

(C) Proportional levels of $eGFP^+hCD45^+$cells in the bone marrow of CTRL and 126KD AML samples after 12 weeks. Data shown represent mean $\pm$ SEM of 4-6 mice.

(D) LSC frequency upon 126KD. Transduced human $CD45^+eGFP^+$ AML cells were flow sorted from primary mice and transplanted into secondary recipients for 8-10 weeks at limiting doses. Human $CD45^+$ marking >0.5% was considered positive for AML engraftment. Human grafts were confirmed to be $CD33^+CD19^-$ AML. Tables depict transplanted cell numbers, engrafted mice percentages and numbers per CTRL and 126KD group for each AML patient sample.

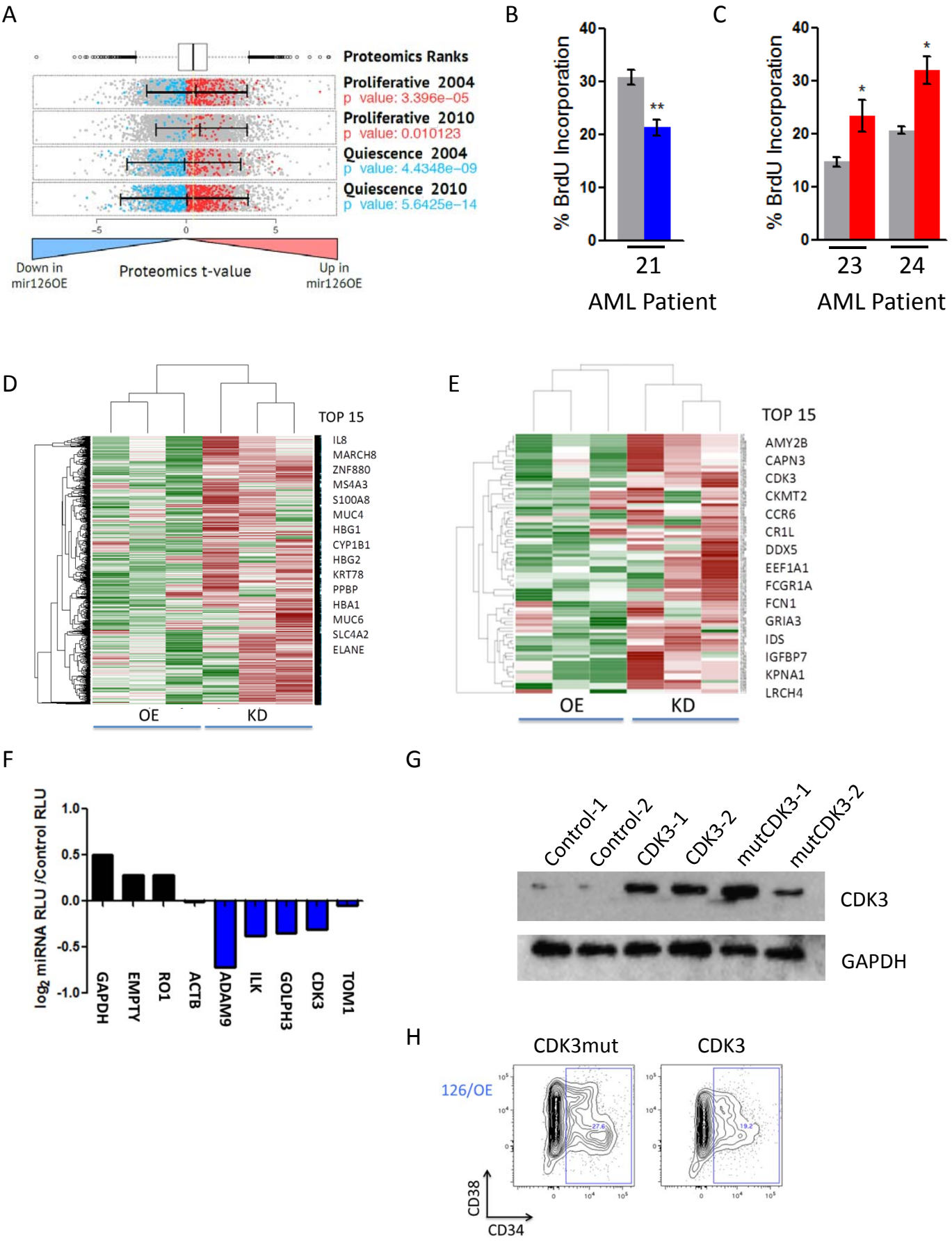
A
Proteomics Ranks
Proliferative 2004
p value: 3.396e−05
Proliferative 2010
p value: 0.010123
Quiescence 2004
p value: 4.4348e−09
Quiescence 2010
p value: 5.6425e−14
−5
0
5
Down in mir126OE
Proteomics t-value
Up in mir126OE
B
% BrdU Incorporation
40
30
20
10
0
**
21
AML Patient
C
% BrdU Incorporation
40
30
20
10
0
*
*
23
24
AML Patient
D
TOP 15
IL8
MARCH8
ZNF880
MS4A3
S100A8
MUC4
HBG1
CYP1B1
HBG2
KRT78
PPBP
HBA1
MUC6
SLC4A2
ELANE
OE
KD
E
TOP 15
AMY2B
CAPN3
CDK3
CKMT2
CCR6
CR1L
DDX5
EEF1A1
FCGR1A
FCN1
GRIA3
IDS
IGFBP7
KPNA1
LRCH4
OE
KD
F
log2 miRNA RLU /Control RLU
1.0
0.5
0.0
-0.5
-1.0
GAPDH
EMPTY
RO1
ACTB
ADAM9
ILK
GOLPH3
CDK3
TOM1
G
Control-1
Control-2
CDK3-1
CDK3-2
mutCDK3-1
mutCDK3-2
CDK3
GAPDH
H
CDK3mut
CDK3
126/OE
27.6
19.2
CD38
CD34

Figure S6, related to Figure 6. miR-126 targets CDK3 in primitive AML cells and enforced expression can rescue the 126OE phenotype in vitro

(A) Comparison of the proteomics data against published quiescent/proliferative signatures. For the proliferative gene sets, Mann Whitney non-parametric analysis was used to test the significance of the overlap between the published proliferative signatures and the proteomic gene set that was up-regulated in miR-126OE. For the quiescent gene sets, Mann Whitney non-parametric analysis was used to look for significance of the overlap between the quiescent signature and proteomic gene set that was down-regulated in miR-126OE. Grey dots represent all the different proteomics ranks t values. Red dots represent proteins found in the gene set of interest with a t-value greater than 0. Blue dots represent proteins found in the gene set of interest with a t value less than 0. The p value indicated below each gene set is the most significant Wilcox p value from the two scenarios tested, "greater" (red), "less" (blue). The yellow dotted line shows the mean of the proteomic ranks t-values.

(B) Human AML samples were thawed, transduced overnight with lentiviruses expressing miR-126 (126OE), a miR-126 sponge (126KD) or an empty vector (CTRL) and transplanted into NSG mice for 8-12 weeks. Sixteen hours prior to mouse euthanization, 1 mg BrdU in 200µl of sterile PBS was IP injected into each mouse. Bone marrow was recovered and processed for analysis. Graph showing proliferation measured by BrdU incorporation assay of CTRL or 126OE transduced AML cells in vivo. Data is shown as mean ± SEM of 5 replicate animals where ** $p<0.01$.

(C) Proliferation measured by BrdU incorporation assay of CTRL or 126KD transduced AML cells in vivo. Data is shown as mean ± SEM of 5 replicate animals where * $p<0.05$.

(D) Heat map ranking all genes that significantly increased in expression with miR-126KD and decreased in expression after miR-126OE in primitive 8227 AML cells. Green indicates decreased expression, red indicates increased expression upon miR-126 modulation. Columns indicate three replicate experiments.

(E) Heatmap of 84 miR-126 predicted targets listed in descending order of level of increase after 126KD. Green indicates decreased expression, red indicates increased expression upon miR-126 modulation. Columns indicate three replicate experiments. Note that CDK3 is near the top of listed targets.

(F) Validation of miR-126 predicted targets by luciferase 3' UTR reporter assay.

(G) Construction and functional validation of CDK3OE and control CDK3mutOE (kinase mutant) vectors. Western blot analysis shows high expression of CDK3 and mutCDK3 7 days post transduction of 8227 cells.

(H) CDK3OE rescue of $CD34^+$ cell expansion upon 126OE. The percentage of $CD34^+$ cells in double transduced cultures is shown as representative flow plots.

Table S4, related to Figure 6. Table S4 is provided as an Excel file

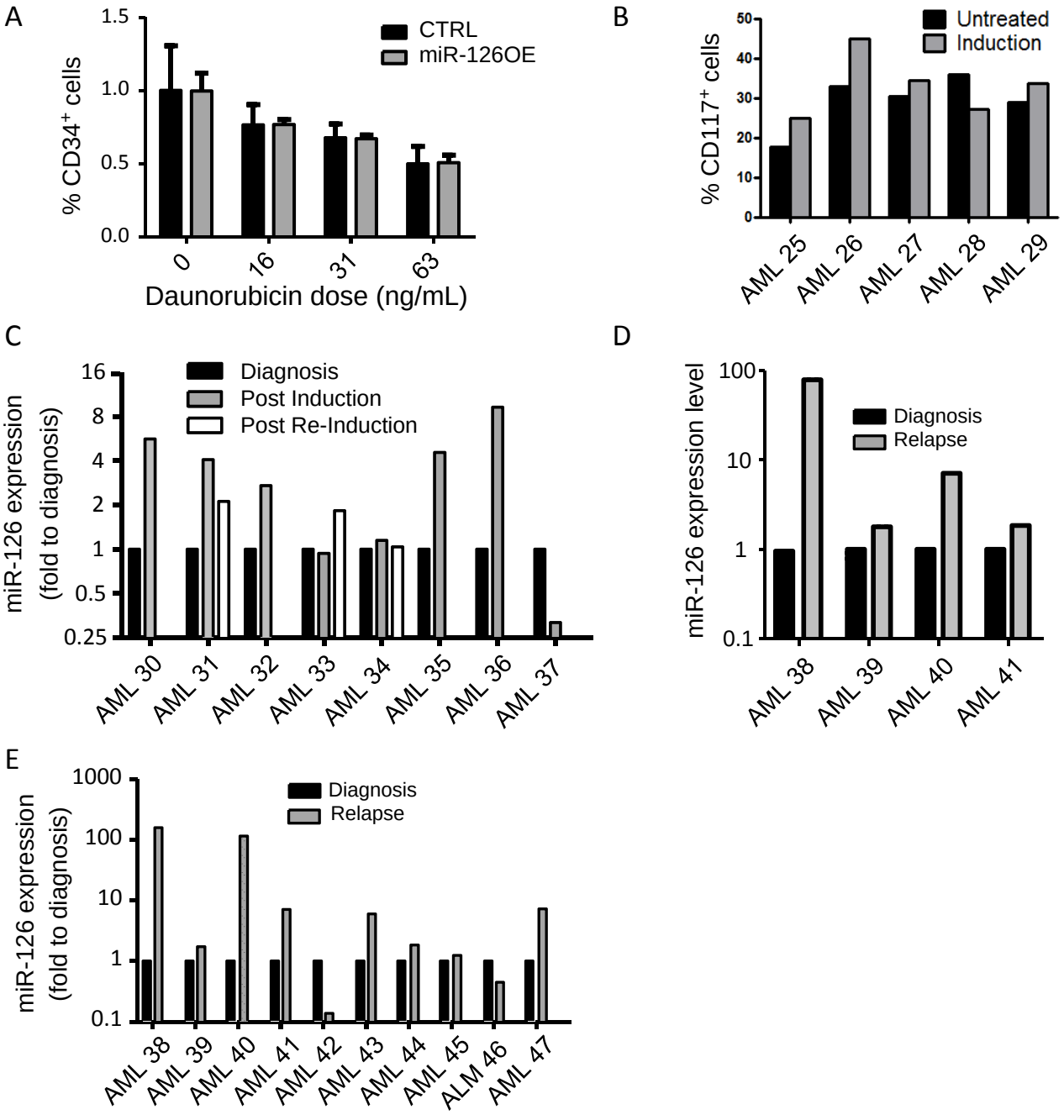
A
% CD34+ cells
1.5
1.0
0.5
0.0
CTRL
miR-126OE
0
16
31
63
Daunorubicin dose (ng/mL)
B
% CD117+ cells
50
40
30
20
10
0
Untreated
Induction
AML 25
AML 26
AML 27
AML 28
AML 29
C
miR-126 expression
(fold to diagnosis)
16
8
4
2
1
0.5
0.25
Diagnosis
Post Induction
Post Re-Induction
AML 30
AML 31
AML 32
AML 33
AML 34
AML 35
AML 36
AML 37
D
miR-126 expression level
100
10
1
0.1
Diagnosis
Relapse
AML 38
AML 39
AML 40
AML 41
E
miR-126 expression
(fold to diagnosis)
1000
100
10
1
0.1
Diagnosis
Relapse
AML 38
AML 39
AML 40
AML 41
AML 42
AML 43
AML 44
AML 45
ALM 46
AML 47

Figure S7, related to Figure 7. High expression of miR-126 protects primitive AML cells from chemotherapy and is a biomarker of refractory LSC.

(A) Graphical representation of non-transduced $CD34^+$ 8227 cells with increasing doses of Daunorubicin. Bars represent mean ± SEM of 3 independent cultures with 10 replicate wells each.

(B) Primary patient AML cells were plated onto MS5 stroma, after 24 hours cells were treated with vehicle or with Daunorubicin/AraC for 72 hours. Cells were recovered and evaluated for live cell content and immuno-phenotype by flow cytometry. Graphical representation of flow cytometry results of chemotherapy treated AML stromal cultures shows an increase in $CD117^+$ AML blasts after 72 hour treatment with Daunorubicin/AraC.

(C) miR-126 levels in 8 individual AML patients who failed to achieve complete remission (CR) after anthracycline/cytarabin induction chemotherapy. qPCR was performed on $CD45^{dim}$ sorted blasts from 8 patient samples at diagnosis (n=8, day 0) and, at day 14 (n=4) and day 30 (n=5) after initiation of induction chemotherapy, as well as on day 30 after (unsuccessful) salvage chemotherapy (n=3).

(D-E) Levels of miR-126 in individual paired diagnosis/relapse AML patient samples. AML patient samples were thawed and sorted for $CD45^{dim}$ or $CD45^{dim}CD117^+$ or $CD45^{dim}CD34^+$ populations. qPCR was used to evaluate the relative levels of miR-126 in paired patient samples. Graphical representation of miR-126 levels in $CD45^{dim}$ (D) and $CD45^{dim}CD117^+$ (samples 38-45) and $CD45^{dim}CD34^+$ (samples 46-47) (E) AML blasts in paired diagnosis/relapse samples.

Table S5, related to Figure 7. Clinical and Molecular Characteristics of AML Patients

AML	Age	Sex	De novo/Secondary	FAB	WBC	Cytogenetics	NPM1 mutation status	FLT3-ITD mutation status
AML Samples used in Stromal Assay								
25	72	M	sAML	n.d.	43	45,X,-Y[20]	n.d.	n.d.
26	28	F	de novo	M1	105.8	46,XX[20]	(+)	(-)
27	58	F	de novo	M5	35.6	46,XX[20]	(+)	(+)
28	50	M	de novo	M4Eo	41.3	46, XY, inv(16)(p13.1q22)[17]/47,idem,+22[3]	n.d.	n.d.
29	67	F	de novo	M1	128	46,XX[20]	n.d.	n.d.
Diagnosis/Refractory AML Patient Sample Pairs								
30	72	M	de novo	M2	41.8	46,XY T(10;13)(q24;q22)[20]	(+)	(+)
31	67	M	sAML	M4Eo	3.7	46,XY[20]	(+)	(-)
32	72	M	de novo	M5B	6.1	Complex/Pseudodiploid	(-)	(-)
33	48	M	de novo	M2	5	46,XY[20]	(-)	(-)
34	42	M	sAML	M2	2.4	Complex	(-)	(-)
35	50	M	de novo	M1	116.9	46,XY[20]	(+)	(+)
36	68	M	de novo	M2	2.1	46,XY[20]	(+)	(-)
37	37	F	de novo	M2	60	46,XX[20]	(+)	(+)
Diagnosis/Relapse AML Patient Sample Pairs								
38	75	M	de novo	M1	60	46,XY[20]	(+)	(-)
39	43	M	de novo	M4Eo	55	46,XY[20]	(+)	(-)
40	60	M	de novo	M4Eo	108	46,XY[20]	(+)	(-)
41	60	M	sAML	M5a	134	45,X,-Y,t(11;19)(q23;p13.1)[20]	n.d.	n.d.
42	27	F	de novo	M2	150	n.d.	(-)	(-)
43	77	M	de novo	M2/M4	215	n.d.	(-)	(+)
44	77	M	de novo	M5	73.9	46,XY[20]	(+)	(+)
45	49	F	de novo	M2	n.d.	46,XX, t(8;11)(q24.2-3;q1)[20]	(+)	(+)
46	72	F	de novo	M4	11.6	46,XX[20]	(-)	(-)
47	52	F	de novo	M4	56.7	46,XX[20]	(-)	(-)

Clinical parameters of the AML patient bone marrow samples used for in vitro stromal chemotherapy assay (AML 19 to AML 23), AML patient cohort that failed to achieve complete remission (AML 24 to AML 31), and paired diagnosis/relapse AML patient samples (AML32 to AML 41).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Limiting dilution analysis

$CD45^+mO^+$ (miR-126OE) or $CD45^+eGFP^+$ (miR-126KD) cells from pooled bone marrow (BM) of primary mice 12 weeks after transplantation and injection of various cell doses into conditioned secondary recipients. A secondary mouse was scored as positive if it had >0.5% BM engraftment 12 weeks after transplantation. Leukemia stem cell (LSC) frequency was estimated by linear regression analysis and Poisson statistics using publicly available ELDA (Extreme Limiting Dilution Analysis, http://bioinf.wehi.edu.au/software/elda/) software (Hu and Smyth, 2009).

Nanostring miRNA data generation and processing

The expression of 30 miRNAs from 74 acute myeloid leukemia (AML) samples from Princess Margaret Cancer Centre (PMCC) were measured and normalized using the NanoString platform (Geiss et al., 2008). For the TCGA cohort, miRNA-Seq RPM (i.e., reads per million) normalized profiles generated using the Illumina Genome Analyzer, which captured the expression of ~492 miRNAs, were downloaded from the TCGA data portal (Cancer Genome Atlas Research Network, 2013). The maximum linear expression of 29 mature miRNAs that were common to both datasets was analyzed in this study. All miRNA expression values were increased by one plus a small normally distributed random value to produce non-zero expression profiles. These profiles were then $\log_2$ transformed and filtered for miRNAs with high variance (i.e., values outside of the 20^{th} to 80^{th} percentile range). Finally, ComBat normalization was applied to reduce the cross-platform differences (i.e., batch effects) between the 2 datasets (Johnson et al.,

2007)(Fei et al., 2013)(Rudy and Valafar, 2011)(Sirinukunwattana et al., 2013). All data analysis was done in R 2.15.2.

Building prognostic miRNA signatures

The glmnet 1.9-3 R package was configured for L1 regularized Cox regression, enabled with leave one out cross validation. This statistical learning tool was then applied to the PMCC dataset to select a minimal weighted combination of miRNA expression that best explained patient survival time. This training phase resulted in a 4-miRNA signature that was tested for prognostic value in the TCGA dataset. Per-patient risk scores were computed using dot products between the expression of signature miRNAs (i.e., c_i) and their corresponding regression coefficients (i.e., weights w_i) as follows: $(w_1 \times c_1) + (w_2 \times c_2) + (w_3 \times c_3) + \ldots$ etc. The resulting continuous scores were further discretized based on the 50th percentile (i.e., median) split, where patients with scores above the split were considered to be at high risk (else low risk) as previously described (Eppert et al., 2011).

miRNA signature performance

In survival analysis, overall survival (OS) was defined as the time from AML diagnosis until death from any cause, event-free survival (EFS) was defined as the time from AML diagnosis until induction failure, relapse, or death from any cause, and relapse-free survival (RFS) defined as the time from the date of first complete remission (CR1) until relapse or death, regardless of cause (Cheson et al., 2003). The survival 2.37-4 package in R (Borgan, 2001) was used to assess the prognostic value of the miRNA signature scores computed for each patient in the TCGA dataset. Survival differences between patients with low- and high- signature scores were assessed using uni- and multi- variate Cox proportional hazards (CPH) and Kaplan-Meier models. In the multivariate case, the prognostic impact of white blood count (WBC), gene mutational status

(i.e., *NPM1*, *FLT3*ITD), age, type of AML onset (i.e., de-novo, secondary), and cytogenetic risk group (i.e., favorable, intermediate, or adverse) served as controls based on their established prognostic relevance in AML and patient data availability (Cancer Genome Atlas Research Network, 2013)(Eppert et al., 2011). The proportional hazards assumption was tested by examining Schoenfeld residuals for each patient parameter (e.g., WBC, age) used in the survival models. Parameters that significantly ($p<0.05$) violated this assumption were used to construct a stratified CPH model to remove their non-proportionality effects on hazard. Signature performance was assessed based on: (1) the lower 95% confidence interval of the signature scores' hazard ratio being greater than the value of one in multivariate survival analysis ($p< 0.05$, Wald test); and (2) the ability of the signature scores to significantly dichotomize low- from high- risk patients in univariate survival models ($p<0.05$, log rank test).

Illumina Microarray

8227 cells were transduced with miR-126/OE or miR-126/KD vectors or their respective empty control lenti-vectors at a multiplicity of infection (MOI) of 30. Cells were expanded under standard culture conditions for two weeks post-transduction and then flow sorted into $CD34^{+}CD38^{-}$, $CD34^{+}CD38^{+}$ and $CD34^{-}CD38^{+}$ populations. Recovered cells were stored in Trizol (Invitrogen) at -80^{o}C. RNA from transduced 8227 cells was extracted using Trizol (Invitrogen) and gene expression assayed on HT-12_v4 microarrays (Illumina). Quantile normalization was performed and probes were filtered by detection p-value (<0.1) (GeneSpring GX, Agilent). Next, to remove uninformative probes, those that did not exceed a threshold of 7.8 in all replicates of any one condition were eliminated, leaving 15812 probes for analysis.

Gene set enrichment analysis

Gene set enrichment analysis was performed using g:Profiler software with the options significant only, ordered query on 2564 genes selected based both on a positive log fold change (logFC) in 8227 126/KD and a negative logFC in 8227 126/OE versus Control samples (Reimand et al., 2011). Gene-sets with a size equal or greater than 500 were removed. Results were visualized using Cytoscape 2.8.1 (Smoot et al., 2011) and an enrichment map (version 1.2 of Enrichment Map software (Merico et al., 2010) was generated using enriched gene-sets with a p-value <0.05 and overlap coefficient set of 0.5.

Mass Spectrometry Sample Preparation

One to two weeks post viral transduction, 8227 cells with miR-126 overexpression (126/OE) and control vector (CTRL) were counted and washed twice with ice-cold PBS. 100,000 cells for each experimental condition, in biological triplicate, were subjected to sample preparation similar to (Kulak et al., 2014). Cells were lysed using 50 µl of lysis buffer (consisting of 6 M Guanidinium Hydrochloride, 10 mM TCEP, 40 mM CAA, 100 mM Tris pH8.5). Samples were boiled at 95^{o}C for 5 minutes, after which they were sonicated on high for 3x 10 seconds in a Bioruptor sonication water bath (Diagenode) at 4^{o}C. Samples were diluted 1:3 with 10% Acetonitrile, 25 mM Tris pH 8.5, LysC (MS grade, Wako) was added in a 1:50 (enzyme to protein) ratio, and samples were incubated at 37^{o}C for 4hrs. Samples were further diluted to 1:10 with 10% Acetonitrile, 25 mM Tris pH 8.5, trypsin (MS grade, Promega) was added in a 1:100 (enzyme to protein) ratio and samples were incubated overnight at 37^{o}C. Enzyme activity was quenched by adding 2% trifluoroacetic acid (TFA) to a final concentration of 1%. Prior to mass spectrometry analysis, the peptides were fractionated using Strong Cation Exchange (SCX) in StageTip format. For each sample, 6 discs of SCX material (3M Empore) were packed in a 200ul tip, and the SCX material activated with 80 µl of 100% Acetonitrile (HPLC grade, Sigma). The tips were

equilibrated with 80 µl of 0.2% TFA, after which the samples were loaded using centrifugation at 4,000x rpm. After washing the tips twice with 100µl of 0.2% TFA, five initial fractions were eluted into clean 500µl Eppendorf tubes using 50, 75, 125, 200 and 300mM ammonium acetate, 20% Acetonitrile, 0.5% formic acid respectively. The final fraction was eluted using 5% ammonium hydroxide, 80% Acetonitrile. The eluted fractions were frozen on dry ice and concentrated in an Eppendorf Speedvac, and re-constituted in 1% TFA, 2% Acetonitrile for Mass Spectrometry (MS) analysis.

Mass Spectrometry Acquisition

For each SCX fraction, peptides were loaded onto a 50cm C18 reverse-phase analytical column (Thermo EasySpray ES803) using 100% Buffer A (0.1% Formic acid in water) at 750bar, using the Thermo EasyLC 1000 µHPLC system in a single-column setup and the column oven operating at 45°C. Peptides were eluted over a 140 minute gradient ranging from 5 to 48% of 100% acetonitrile, 0.1% formic acid at 250 nl/min, and the Orbitrap Fusion (Thermo Fisher Scientific) was run in a 3 second MS-OT, ddMS2-IT-HCD top speed method. Full MS spectra were collected at a resolution of 120,000, with an AGC target of 4×10^5 or maximum injection time of 50ms and a scan range of 400–1500m/z. Ions were isolated in a 1.6m/z window, with an AGC target of 1×10^4 or maximum injection time of 50ms, fragmented with a normalized collision energy of 30 and the resulting MS2 spectra were obtained in the ion trap. Dynamic exclusion was set to 60 seconds, and ions with a charge state <2, >7 or unknown were excluded. MS performance was verified for consistency by running complex cell lysate quality control standards, and chromatography was monitored to check for reproducibility. Each sample was run in technical duplicate and biological triplicate, and the reproducibility of the analyses is depicted in **Table S4**. The mass spectrometry data have been deposited to the ProteomeXchange

Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD001994 (username: reviewer02600@ebi.ac.uk, password: aLahIn44)(Hermjakob and Apweiler, 2006).

Label-free Quantitative Proteomics Analysis

The raw files were analyzed using MaxQuant version 1.5.2.8 (Cox and Mann, 2008) and standard settings. Briefly, label-free quantitation (LFQ) was enabled with a requirement of 3 unique peptides per protein. Variable modifications were set as Oxidation (M), Acetyl (protein N-term), Gln->pyro-Glu and Glu->pyro-Glu. Fixed modifications were set as Carbamidomethyl (C), false discovery rate was set to 1% and "match between runs" was enabled. The resulting protein groups file, containing all the LFQ intensities across all the samples was processed in Perseus (filtering for contaminants and reverse hits), resulting in 8,848 proteins identified in total, and 4,837 proteins quantified across all samples. To determine those proteins that are significantly different between 126OE and CTRL samples, the ratios of 126OE vs CTRL were calculated in each biological repeat and subjected to statistical analysis in Limma (R Statistical Framework) with Benjamini-Hochberg adjustment. This table is included as **Table S4**, and was used as input for downstream analysis with GSEA.

Proteomics

MaxQuant LFQ (Cox and Mann, 2008) intensities were used as a measure of protein expression in 8227 126OE and control samples. The entire protein expression set consisting of 3 biological replicates for each treatment group and corresponding technical replicates (total of 12 samples) was quantile normalized in R (R version 3.1.1) using the normalizer package (version 1.0). The normalized protein expression was further filtered to contain only proteins that had at least two measurements in either treatment or control.

Difference in protein expression between the groups was assessed using a moderated t-test available in the bioconductor limma package (version 3.20.9). P-values were further corrected to control for multiple hypothesis testing using the Benjamini-Hochberg procedure. 638 proteins had significant differential expression with nominal p-value <0.05, of those 451 were upregulated (t>0) and 187 were downregulated (t<0). Proteins and their corresponding t-statistic were used to create a rank file to be used in pathway analysis described below.

Pathway Analysis on the proteomics data

Gene Set Enrichment Analysis (Subramanian et al., 2005) was performed using the protein expression ordered from largest to smallest t statistics with parameters set to 1000 gene-set permutations and gene-sets size between 5 and 500. The gene-sets included for the GSEA analyses were obtained from KEGG, MsigDB-c2, NCI, Biocarta, IOB, Netpath, HumanCyc, Reactome, Panther and Gene Ontology (GO) databases, updated December 24, 2014 (http://download.baderlab.org/EM_Genesets/). An enrichment map (version 2.1.0 of Enrichment Map software (Merico et al., 2010) was generated using Cytoscape 3.2.1 using significantly enriched gene-sets with a nominal p-value <0.05 and FDR <0.01. Similarity between gene-sets was filtered by Jaccard coefficient >0.25. Only gene-sets enriched in downregulated proteins were further analyzed and visualized.

Correlation between miR-126 predicted targets and the proteomics modulated pathway

Four databases were used to create a list of miR-126 predicted targets (DIANA microT, picTar, TargetScan from the miRbase website (http://www.mirbase.org)) and miRanda from the microCosm website (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/). Downregulated predicted targets were compared to enriched gene-sets in the enrichment map and significance of overlap was scored using 2 statistical tests (a hypergeometric test and a one-

sample Wilcoxon rank sum test) so that highest significance corresponds to higher number of genes in each overlap relative to gene-set size (hypergeometric test) and proteins in the overlap showing the highest differential expression amplitude (one sample Wilcoxon test).

Correlation between the transcriptomic data and the proteomic modulated pathways

2564 genes were selected based both on a positive log fold change (logFC) in 8227 126KD and a negative logFC in 8227 126OE versus control samples. The list was ranked by the 8227 126KD logFC in a decreasing order and compared to enriched gene-sets in the enrichment map and significance of overlap was scored using 3 statistical tests (one hypergeometric test, two one-sample Wilcoxon rank sum tests) so that highest significance corresponds to a higher number of genes in each overlap relative to gene-set size (Hypergeometric test), genes in the overlap showing higher differential expression amplitude (one sample Wilcoxon test), and proteins in the overlap showing higher differential expression amplitude (one sample Wilcoxon test).

Correlation of the miR-126 predicted targets and the miR-126 modulated pathways

Four databases were used to create a list of miR-126 predicted targets (DIANA microT, picTar, TargetScan from the miRbase website (http://www.mirbase.org)) and miRanda from the microCosm website (http://www.ebi.ac.uk/enright srv/microcosm/htdocs/targets/v5/). 84 predicted targets were part of the 2564 genes up-regulated in 8227 126KD and down-regulated in 8227 126OE. This list was compared to enriched gene-sets in the enrichment map and overlap was scored using Fisher's Exact Test p-value. Overlaps with p-value <0.05 were visualized.

Global miRNA Profiling

As described in (Eppert et al., 2011), cells from 16 AML patients (**Figure S1A**) were stained with antibodies to cell surface markers CD34 and CD38 and flow sorted into $CD34^{+}CD38^{-}$,

$CD34^+CD38^+$, $CD34^-CD38^+$ and $CD34^-CD38^-$ populations. Three independent pooled cord blood samples from 15–22 donors were used for isolation of HSC subsets and progenitors. Representative sorting gates are in **Figure 1A**.

NSG mice 8–13 weeks old were pretreated with 2.75–3.4 Gy radiation before being injected intra-femorally with AML cells at a dose of 200 to 2.87×10^6 sorted cells per mouse, as described (Eppert et al., 2011). Mice were killed at 12 weeks (mean 10 weeks), and bone marrow from the injected right femur, opposite femur and, in some cases, both tibias and spleen were collected for flow cytometry and secondary transplantation. Human engraftment was evaluated by flow cytometry of the injected right femur and non-injected bones and spleen. A threshold of 0.5% human $CD45^+$ cells in bone marrow was used as positive for human engraftment. Secondary transplantation was done by intrafemoral injection of cells from either right femur or pooled bone marrow from primary mice into one to three secondary mice pretreated with irradiation and antibody to CD122.

miRNA labeling

Target preparation was done according to (Lu et al., 2005). Briefly, two synthetic pre-labeled control nucleotides (5' – pCAGUCAGUCAGUCAGUCAGUCAG-3', and 5'-pGACCUCCAUGUAACGUACAA-3', Dharmacon) were spiked at 3fmoles per μg of total RNA to control for target preparation control. Small RNA's were recovered for 1-10 μg total RNA by PAGE purification and adaptor ligated sequentially on the 3' end and the 5' end using T4 RNA ligase (Amersham Biosciences). After reverse transcription using adaptor specific primers, products were PCR amplified for 18 cycles for 10 μg starting total RNA using 3' – primer 5'-TACTGGAATTCGCGGTTA-3' and 5' primer-biotin-CAAACGAATTCCTCACTAA-3'(IDT). PCR products were precipitated and dissolved in 66

μl TE buffer (10 mM Tris-HCL pH 8.0, 1 mM EDTA) containing two biotinylated post-labelling control oligonucleotides (100 fmoles of FVR506, 25 fmoles PTG20210).

Bead based detection

As described in (Lu et al., 2005), miRNA capture probes were conjugated to carboxylated xMAP beads (Luminex Corportion) in a 96-well plate. Samples were hybridized in a 96 well plate, with 2 mock PCR samples in each plate as a background control. Hybridization was carried out overnight. Beads were spun down, resuspended in 1x TMAC containing 10 $\mu g/mL^{-1}$ streptavividin-phycoerythrin (Molecular probes) before data acquisition of a Luminex 1001S machine. Median fluorescence intensity values were measured.

miRNA array computational analysis

Profiling data was scaled to the post-labelling controls and then the pre-labelling controls, in order to normalize readings from different probe/bead sets for the same sample and to normalize for the labelling efficiency, respectively. Data were threshholded at 32 and $\log_2$ transformed. The LSC miRNA signature was generated using a Smyth's moderated t-test with Benjamini-Hochberg multiple testing correction to compare fractions positive for LSCs versus those without LSCs.

Luciferase Reporter assay

Luciferase assay (Switchgear Genomics) was performed according to the manufacturers' protocol. Briefly, 293T cells were seeded to 40% confluency in 100 μl total volume in 96 well white TC plates (NUNC). The next day, equal volumes of mixture 1 (GoClone reporters; 30 ng/μl, miR-126 mimic or non-targeting mimic; 100 nM, and serum free media) was combined with mixture 2, (Dharmafect Duo/serum-free media) and incubated at room temperature RT for

20 minutes. After incubation, 4 volumes of pre-warmed serum-free media were added and mixed. 100 μl of the mixture was added to each well containing 293T cells and incubated overnight. Reconstituted luciferase substrate (100 μl) was added to each well, incubated at RT for 30 minutes and read for 2 seconds on a spectraMAX luminometer. Knockdown was determined by calculating the luciferase signal ratio for each reporter construct for miR-126 over the non-targeting control miRNA. GoClone control reporters used were GAPDH-3'UTR, ACTB-3'UTR, EMPTY-3'UTR, R01, and gene specific reporters were ADAM9-UTR, ILK-UTR, GOLPH3-3'UTR, CDK3-3'UTR and TOM1-3'UTR.

AML Stromal Chemotherapy Assay

Low passage MS5 stromal cells were seeded into 0.1% gelatin coated 96 well tissue culture plates and cultured for 48 hours in H5100 media (SCF; 100 ng/ml, TPO; 50 ng/ml. IL-7; 20 ng/ml, IL-3; 10 ng/ml, IL-6; 20 ng/ml, FLT3L; 10 ng/ml, G-CSF; 20 ng/ml, GM-CSF; 20 ng/ml) with 1% penicillin/streptomycin. After two days, primary patient AML cells (depleted for human T cells; $1x10^5$/well) were plated onto MS5 stroma and cultured overnight. After 24 hours, cells were treated with vehicle or with Daunorubicin/AraC (1:1 ratio; 50 ng/mL Daunorubicin and 500 ng/mL Ara-C) for 72 hours. Cells were recovered and evaluated for live cell content and immuno-phenotype by flow cytometry. Total RNA was recovered for qPCR detection of miR-126 levels.

Intracellular and Phosphoflow

Cultured 8227 cells were washed twice with 1x PBS and pelleted to eliminate culture medium. After washing, cells were stained for CD34 and CD38 cell surface antigens for 20 minutes at RT. Cells were washed and pelleted. Cells were immediately fixed with paraformaldehyde (final

concentration: 1.6%) for 10 minutes at room temperature. Cells were then centrifuged, washed once with PBS 1% BSA to remove residual PFA and permeabilized with ice-cold Perm buffer III (BD Phosflow for 30 min at 4°C followed by 2 washes in order to remove traces of methanol). Intracellular antibody staining was then performed. Phospho-Rb Ser807/811 was detected by a monoclonal antibody (clone D20B12; Cell Signaling), CDK3 was detected by a polyclonal antibody (clone D01P; Abnova) and detected by a donkey anti-rabbit brilliant violet 421 conjugated secondary antibody. Staining was performed by incubating permeabilized cells with the phospho-specific antibody for 30 minutes on ice and at dark, diluted in PBS 1% BSA at a final concentration of 1:50. After incubation, cells were washed in PBS 1% BSA and then stained in secondary antibody for 30 minutes at a final concentration of 1:50. Cells were analyzed by multi-parameter flow cytometry.

Western blot analysis

8227 cells were cultured as described and transduced with viral particles at a multiplicity of infection (MOI) of 30 for at least 16 hours. Cells were allowed to expand for 14 days and then flow sorted for mO^+ cells. Total cellular proteins were extracted with RIPA buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) supplemented with protease and phosphatase inhibitors: 1 mM PMSF, 10 mM NaF, 1 mM Na_3VO_4, CompleteMini™ and PhosStop ™ (Roche). Samples were resuspended in the lysis solution and incubated at 4°C for 30 minutes. Cell lysates were cleared by centrifugation at 10,000×g for 10 minutes at 4°C, and the supernatants were collected and assayed for protein concentration using Lowry assay based method (DC, BioRad). 40-50 micrograms of proteins were run on SDS-PAGE under reducing conditions. For immunoblotting, proteins were transferred to PVDF membranes, incubated with the specific antibody (anti-ADAM9 Cell Signaling #2099 1:1000, and ant-pan AKT Cell

Signaling # 4685 1:500, anti-PIK3R2 Cell Signaling #4257 1:1000, and GAPDH 1:10,000, Sigma) followed by peroxidase-conjugated secondary antibodies. Bands were visualized on Kodak BioMax film.

Lentiviral constructs, cell culture and colony formation

Lentiviral vector platforms for ectopic miRNA expression, stable knockdown and live cell miR-reporting were described previously (Gentner et al., 2010). Third generation lentiviral vector particles pseudotyped with VSV-G were generated as described (Guenechea et al., 2000).

8227 cell cultures were initiated from a primary patient sample with outgrowth after 1 month; this stock was used for all experiments. Primitive $CD34^+CD38^-$ 8227 cells were flow sorted and plated in culture media (described in Supplementary Experimental Procedures) for viral transduction and initiation of a new culture that re-establishes the cellular hierarchy. One to two weeks post viral transduction, cultures were re-sorted for $CD34^+CD38^-$ (LSC enriched), $CD34^+CD38^+$ (leukemia progenitor enriched) and $CD34^-CD38^+$ (mature AML blasts) populations for experimental culture studies or colony assays. Individual cultures were passaged weekly and monitored with phenotypic cell surface markers.

For chemotherapy experiments, $1x10^5$ transduced 8227 cells were plated into 96-well plates, exposed the following day to a dose range of Daunorubincin and analyzed using CD34 and CD38 cell surface markers 72 hours later. $CD34^+$ levels were normalized to vehicle only wells.

Ki67 and Hoechst flow cytometry

Cells were stained for surface markers, washed and fixed using BD Cytofix buffer, washed and permeabilized with BD Perm 2 (BD), washed and stained with PE- or FITC- or PerCP-Cy5.5

conjugated Ki67 antibody (BD) and finally resuspended in BD Cytofix buffer with Hoechst at 1 μg/mL. The cells were then analyzed on a BD LSRII machine with a UV laser.

Quantitative PCR

miR-126 expression was analyzed as described (Lechman et al., 2012). Briefly, small RNAs were extracted using Trizol (Life Technologies) and miRNA expression levels were determined by the Applied Biosystems Taqman® microRNA Assay system. Reactions were carried out in triplicate in an ABI Prism 7900HT (Applied Biosystems, Foster City, CA). miRNA expression was normalized to RNU48.

Statistical analysis

Unless otherwise indicated, mean $\pm$ SEM values are reported in the graphs. For pairwise comparisons, a Mann-Whitney non-parametric test was used unless otherwise indicated.

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Myeloid Malignancies

John Howard, M.D.
Administrator, World Trade Center Health Program

February 1, 2014

Executive Summary

Beginning on February 1, 2014, the World Trade Center (WTC) Health Program will consider blood or bone marrow disorders of the myeloid line to be slow-growing blood cancers. Accordingly, they will be considered WTC-related health conditions, making them available for WTC Health Program medical treatment services for eligible members. These cancers had been considered non-malignant by the Administrator because they were referred to as "pre-leukemic" hematopoietic disorders in the medical literature. Recent scientific advances, however, characterize these "pre-leukemic" myeloid neoplasms as slow-growing blood cancers, and authoritative scientific sources now consider them to be malignant myeloid neoplasms.

After receiving a request from the WTC Clinical Centers of Excellence to review certain myeloid disorders in terms of their status as malignancies,[1] the WTC Health Program has determined that, in addition to types of leukemias, these myeloid malignancies are eligible for coverage by the WTC Health Program as WTC-related health conditions.[2] The group of myeloid malignancies includes the following health conditions:

(1) Myelodysplastic Syndromes (MDSs);
(2) Myeloproliferative neoplasms (MPNs);
(3) Myelodysplastic/myeloproliferative neoplasms (MDS/MPN); and
(4) Myeloid malignancies associated with eosinophilia and abnormalities of growth factor receptors derived from platelets or fibroblasts.[3]

[1] Letter to Drs. Dori Reissman and John Halpin of the WTC Health Program from World Trade Center Clinical Centers of Excellent Principal Investigators dated December 16, 2013 (on file at WTC Health Program).

[2] See 42. C.F.R. § 88.1 (Table 1).

[3] Acute myeloid leukemia (AML) remains eligible for coverage as a WTC-related health condition because it is already included in the List of WTC-Related Health Conditions. See 42 C.F.R. § 88.1 (Table 1).

I. Introduction

In December of 2013 the WTC Clinical Centers of Excellence (CCEs) requested that the WTC Health Program review certain myeloid disorders in terms of their status as malignancies.[1] MDS is one type of a group of myeloid malignancies. Therefore, based on the CCEs' request, the Administrator reviewed the available scientific literature and authoritative disease classification sources pertaining to the malignancy of myeloid neoplasms.

The term "myeloid" includes all cells belonging to the granulocyte (i.e., neutrophil, eosinophil, basophil), monocyte/macrophage, erythroid, megakaryocyte, and mast cell lineages. Myeloid malignancies are clonal diseases of hematopoietic stem or progenitor cells.[4] These malignancies can be present in the bone marrow and peripheral blood. They result from genetic and epigenetic alterations that perturb key processes such as self-renewal, proliferation and impaired differentiation.[5,6]

Some myeloid disorders, such as the myeloid leukemias, have long been considered malignant while other myeloid disorders have been considered non-malignant or pre-leukemia blood disorders which may become malignant over time. However, recent scientific findings indicate that these "pre-leukemia" blood disorders are actually forms of slow-growing blood cancers. [7,8]

Based on the morphology, cytochemistry, immunophenotype, genetics, and clinical features of myeloid disorders, the World Health Organization (WHO) categorizes myeloid malignancies into five primary types: (1) acute myeloid leukemia; (2) myelodysplastic syndromes (MDS); (3) myeloproliferative neoplasms (MPN); (4) myelodysplastic and myeloproliferative (MDS/MPN) neoplasms; and (5) myeloid neoplasms associated with eosinophilia and abnormalities of growth factor receptors derived from platelets or fibroblasts. The types and subtypes of myeloid malignancies are identified in Table 1 in the Appendix.

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II. Risk Factors

A. Myelodysplastic Syndrome and Myeloproliferative Neoplasms

The primary risk factor for MDS is age. The majority of secondary MDS cases occur after treatment for other cancers with radiation therapy or chemotherapy that employs alkylating agents or topoisomerase inhibitors. In addition, several environmental and/or occupational exposures have been associated with increased rates of MDS or cytogenetic abnormalities associated with MDS including pesticides[9], benzene[10], organic solvents[11], semi-metals[12], and inorganic dusts[13].

Studies of occupations identified increased incidence of MPN among poultry workers, commercial pressmen, petroleum refinery workers, agricultural workers, cooks/waiters and clerks. Studies of associations with exposure to chemicals such as benzene, petroleum solvents, hair dyes, and pesticides have produced inconsistent results.[14]

B. Myeloid Malignancies Other Than AML, MDS and MPN

Information on associations of environmental or occupational exposures for other myeloid malignancies was not found in a Medline search of the relevant medical literature.

[9] Vundinti BR, Kerketta L, Jijina F, Ghosh K [2009]. Cytogenetic study of Myelodysplastic syndrome from India. Indian J Med Res 130:155-159.

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III. Clinical and Pathologic Features

The clinical and pathologic features of myeloid malignancies vary according to type.

Acute myeloid leukemia. AML results from the clonal expansion of myeloid blasts in the peripheral blood, bone marrow or other tissue. It is caused when either the myeloid stem cells produce abnormal myeloblasts which do not become healthy white blood cells or too many myeloid stem cells become abnormal red blood cells or platelets. As a result, leukemic blasts, or immature cell forms, accumulate in the bone marrow, peripheral blood, and occasionally in other tissues, and the production of normal red blood cells, platelets, and mature granulocytes are reduced a variable amount. The increased production of malignant cells, along with a reduction in these mature elements, results in a variety of systemic consequences including anemia, bleeding, and an increased risk of infection.[13]

Myelodysplastic syndromes. MDSs are a spectrum of bone marrow failure disorders that share the common pathologic feature of cytological dysplasia. They progress to acute myeloid leukemia (AML) in about 30% of patients. MDSs are classified according to features of cellular morphology, cellular and molecular genetics, immunophenotyping, etiology, and clinical presentation. The seven subtypes of MDSs are listed in Table 1 in the Appendix.

The morphological classification of MDSs is largely based on the percent of myeloblasts in the bone marrow and blood, the type and degree of myeloid dysplasia, and the presence of ring sideroblasts. MDSs remain among the most challenging of the myeloid malignancies to diagnose and classify, particularly in cases in which the blast percentage is not increased in the peripheral blood or bone marrow.[13]

Myeloproliferative Neoplasms. MPNs are clonal hematopoietic stem cell disorders characterized by proliferation of one or more of the myeloid lineages. The subtypes of myeloproliferative malignancies are identified in Table 1.

Each of these disorders involves dysregulation at the multipotent hematopoietic stem cell (CD34) and clonal myeloproliferation and the absence of dyserythropoiesis, dysgranulopoiesis and monocytosis. Abnormal proliferation among this type arises from specific genetic rearrangements or mutations affecting protein tyrosine kinases or related molecules which produce constitutively active signal transduction pathways.[15,16]

[15] Kittur J, Knudson RA, Lasho TL, Finke CM, Gangat N, Wolanskyj AP, Li C, Wu W, Ketterling RP, Pardanani A, Tefferi A [2007]. Clinical correlates of JAK2V617F allele burden in essential thrombocythemia. Cancer 109:2279–2284.

[16] De Keersmaecker K, Cools J [2006]. Chronic myeloproliferative disorders: a tyrosine kinase tale. Leukemia. 20:200–205.

Among the subtypes of MPN, chronic myelogenous leukemia (CML) is defined by its causative molecular lesion, the BCR-ABL fusion gene, which most commonly results from the Philadelphia translocation (Ph). Polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) are the three main Ph-negative myeloproliferative neoplasms. The cardinal features of the main myeloproliferative neoplasms are an elevated white blood cell count in CML, increased red-cell mass in PV, a high platelet count in ET, and bone marrow fibrosis in PMF.[13,17]

Myelodysplastic/myeloproliferative neoplasms. MDS/MPNs are clonal myeloid disorders that possess both dysplastic and proliferative features but are not properly classified as either myelodysplastic syndromes (MDS) or chronic myeloproliferative disorders (CMPD). The MDS/MPN category includes myeloid neoplasms with clinical, laboratory, and morphologic features that overlap MDS and MPN. These disorders commonly have mutations in the genes that encode the RAS or MAPK dependent signaling pathways.[13]

Myeloid neoplasms associated with eosinophilia and abnormalities of growth factor receptors derived from platelets or fibroblasts. These malignancies arise by forming abnormal fusion genes that encode altered surface or cytoplasmic proteins that activate signal transduction pathways.[13,18] The subtypes of myeloid neoplasms associated with eosinophilia and abnormalities of platelet or fibroblast growth factor receptors are listed in Table 1 in the Appendix. Although eosinophilia is characteristic of each subtype, the clinical presentation of each subtype varies.[13,19]

IV. Classification

The WTC Health Program uses the International Classification of Diseases Version 9 (ICD-9) coding system for carcinogenic and non-carcinogenic health condition classification. Under the ICD-9 coding system, myelodysplastic and myeloproliferative neoplasms are considered pre-leukemia blood disorders, and therefore were not considered by the WTC Health Program as malignancies. However, since the ICD-9 coding system was developed by the WHO, substantial scientific progress has been made in understanding the behavior of these malignancies. As a result, these health conditions—formerly classified not to be malignancies — are now consider to be

[17] Anastasi J [2009]. The myeloproliferative neoplasms: insights into molecular pathogenesis and changes in WHO classification and criteria for diagnosis. Hematol Oncol Clin North Am 23(4):693-708.

[18] Savage N, George TI, Gotlib J [2013]. Myeloid neoplasms associated with eosinophilia and rearrangement of PDGFRA, PDGFRB, and FGFR1: a review. Int J Lab Hematol 35(5):491-500.

[19] Gotlib J, Cools J [2008]. Five years since the discovery of FIP1L1-PDGFRA: what we have learned about the fusion and other molecularly defined eosinophilias. Leukemia 22(11):1999-2010.

malignancies in the newer coding systems such as the International Classification of Diseases for Oncology (ICD-O), which is used for cancer classifications by cancer registries and by the ICD-9 replacement, ICD-10.

In 2000, the World Health Organization (WHO) changed the behavior code for myelodysplastic and myeloproliferative conditions in the ICD-O from 1 (i.e., "uncertain whether benign or malignant") to 3 (i.e., "malignant"). Based on the underlying science that led to the changes in the ICD-O coding system, these neoplasms became reportable to population-based cancer registries, such as the Surveillance, Epidemiology, and End Results (SEER) Program in 2001.

In 2008, the WHO updated the *Classification of Tumours of the Haematopoietic and Lymphoid Tissues*, a worldwide consensus of hematologic malignancies. The WHO classification system uses the available information on morphology, cytochemistry, immunophenotype, genetics, and clinical features to define clinically meaningful diseases. In this classification system myeloid neoplasms are characterized as malignant.

In making decisions on coverage of myeloid neoplasms, the WTC Health Program is decreasing its reliance on older disease classification systems, such as the ICD-9 coding system, and increasing its reliance on newer authoritative sources, such as ICD-O and ICD-10 coding systems. In addition the WTC Health Program is increasing its reliance on mature scientific information available in the published literature whose significance has been widely acknowledged. This changed emphasis will allow the WTC Health Program to make certification decisions based on more current scientific information.

V. Incidence of Myeloid Malignancies

Acute myeloid leukemia (AML) is the most common acute leukemia in adults and accounts for approximately 80 percent of cases in this group.[20] The incidence increases from about 1.3 per 100,000[21] for those under to 65 to about 12.2 cases per 100,000 for those over 65 years [Siegel 2012]. The age-adjusted incidence rate for acute myeloid leukemia in the years 1975–2003 was 3.7 per 100,000 persons.[22,23]

[20] In children less than 10 years of age AML accounts for less than 10 percent of acute leukemias.

[21] All incidence rates reported here are age-adjusted to the 2000 U.S. Standard Population (19 age groups-Census P25-1130).

[22] SEER 9 areas (San Francisco, Connecticut, Detroit, Hawaii, Iowa, New Mexico, Seattle, Utah, and Atlanta).

[23] Howlader N, Noone AM, Krapcho M, Garshell J, Neyman N, Altekruse SF, Kosary CL, Yu M, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA (eds). SEER Cancer Statistics Review, 1975-2010,

The other myeloid neoplasms occur at much lower frequencies, but individual incidence rates for each type have not been published. The combined age-adjusted incidence rate for MDSs, MPNs and chronic myelomonocytic leukemia for 2006-10 was 7.8 per 100,000 persons.[22,24] All other myeloid malignancies are very rare, and their precise incidence is unknown at this time.

However, based on the reported incidence of the malignant myeloid malignancies in general, it is reasonable to assume that the combined age-adjusted incidence rate for all myeloid malignancies in the U.S. is less than 15 per 100,000 persons.

VI. Summary of Evidence

Recent scientific advances and authoritative classification sources characterize myeloid neoplasms as slow-growing blood cancers or malignancies. Based on this evidence, the WTC Health Program considers MDSs, MPNs, MDS/MPN, and myeloid neoplasms associated with eosinophilia and abnormalities of growth factor receptors derived from platelets or fibroblasts, to be eligible for coverage under the rare cancers category of covered WTC-related health conditions. Acute myeloid leukemia (AML) is eligible for coverage because it is included in the List of WTC-related health condition.

VII. Certification of Myeloid Malignancies for WTC Health Program Coverage

The WTC Health Program bases coverage decisions for cancer on the WHO disease classification systems. However, classification systems change over time, and even the latest classification systems may not be based on the most recent scientific evidence and diagnostic criteria that contribute to identifying conditions. Consequently, the WTC Program Administrator has determined that reliance on classification systems can inappropriately constrain the decisions of the WTC Health Program and can result in undesired denial of coverage.

Two conditions which have been affected by the reliance on classification systems are Myelodysplastic Syndromes (MDS) and Myeloproliferative Neoplasms (MPN). These disorders had been considered pre-leukemia blood disorders and were not classified as malignant conditions. However, they demonstrate clonal proliferation behavior which is

National Cancer Institute. Bethesda, MD, http://seer.cancer.gov/csr/1975_2010/, based on November 2012 SEER data submission, posted to the SEER web site, April 2013.

[24] SEER 18 areas (San Francisco (SF), Connecticut, Detroit, Hawaii, Iowa, New Mexico, Seattle, Utah, Atlanta (ATL), San Jose-Monterey (SJM), Los Angeles (LA), Alaska Native Registry, Rural Georgia (RG), California, excluding SF/SJM/LA, , Kentucky, Louisiana, New Jersey and Georgia excluding ATL/RG).

a characteristic used to distinguish between benign and malignant conditions, and the scientific evidence indicates they should be considered forms of slow-growing blood cancers.

To inform certification decisions of myeloid neoplasms the WTC Health Program will use authoritative sources, such as the WHO disease classification system, and mature scientific information available in the published literature whose significance has been widely acknowledged. The WTC Health Program considers MDS, MPN, MDS/MPN, and myeloid neoplasms associated with abnormalities of growth factor receptors to be malignant cancers which can be covered under the rare cancers category of covered WTC-related health conditions. AML is listed as a covered WTC-related health condition.

When a physician from the Clinical Center of Excellence, or the Nationwide Provider Network, determines a neoplasm to be a "myeloid malignancy", the WTC Health Program will consider the myeloid malignancy for certification as a type of leukemia or as a "rare cancer."

Each determination of a myeloid neoplasm/malignancy as a WTC-related health condition must be considered for certification under (1) the minimum latency requirements for lymphoproliferative and hematopoietic cancers (including all types of leukemia and lymphoma),[25] and (2) the exposure requirements specified by the WTC Health Program in the WTC-3 Certification Request form.

[25] WTC Health Program. *Minimum Latency & Types or Categories of Cancer* (May 1, 2013). http://www.cdc.gov/wtc/pdfs/wtchpminlatcancer2013-05-01.pdf

APPENDIX

Table 1. The 2008 World Health Organization classification scheme for myeloid neoplasms .[A]

1. Acute myeloid leukemia

2. Myelodysplastic syndromes (MDS)

- 2.1 Refractory cytopenia with unilineage dysplasia
  - Refractory anemia; Refractory neutropenia; Refractory thrombocytopenia
- 2.2 Refractory anemia with ring sideroblasts
- 2.3 Refractory cytopenia with multilineage dysplasia
- 2.4 Refractory anemia with excess blasts-1
- 2.5 Refractory anemia with excess blasts-2
- 2.6 Myelodysplastic syndrome with isolated del(5q)
- 2.7 Myelodysplastic syndrome, unclassifiable

3. Myeloproliferative neoplasms (MPN)

- 3.1 Chronic myelogenous leukemia
- 3.2 Polycythemia vera
- 3.3 Essential thrombocythemia
- 3.4 Primary myelofibrosis
- 3.5 Chronic neutrophilic leukemia
- 3.6 Chronic eosinophilic leukemia, not otherwise categorized
- 3.7 Hypereosinophilic syndrome
- 3.8 Mast cell disease
- 3.9 MPNs, unclassifiable

4. Myeloplastic/myeloproliferative neoplasms (MDS/MPN)

- 4.1 Chronic myelomonocytic leukemia
- 4.2 Juvenile myelomonocytic leukemia
- 4.3 Atypical chronic myeloid leukemia
- 4.4 MDS/MPN, unclassifiable

5. Myeloid neoplasms associated with eosinophilia and abnormalities of PDGFR-A or -B, or FGFR1

- 5.1 Myeloid neoplasms associated with PDGFRA rearrangement
- 5.2 Myeloid neoplasms associated with PDGFRB rearrangement
- 5.3 Myeloid neoplasms associated with FGFR1 rearrangement
  - (8p11 myeloproliferative syndrome)

[A] Adapted from: Tefferi A, Vardiman JW [2008]. Classification and diagnosis of myleoproliferative neoplasms: the World Health Organization criteria and point-of-care diagnostic algorithms. *Leukemia* 22:14-22.

Karar Tarihi	Toplantı Sayısı	Karar Sayısı
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Tıbbi Biyoloji Anabilim Dalı Başkanlığından gelen 29.06.2016 tarih ve 153761 sayılı tez önerisi konulu yazı üzerinde görüşüldü.

Enstitümüz Tıbbi Biyoloji Anabilim Dalı yüksek lisans öğrencilerinden Cemile **ERSÖZ**'ün **"Myeloid Malignansilerde Hematopoetik miRNA EkspresyonlarınınAraştırılması"** başlıklı yüksek lisans tez önerisinin; "Mersin Üniversitesi Lisansüstü Eğitim-Öğretim ve Sınav Yönetmeliği" nin 24. maddesi uyarınca kabul edilmesine, durumun Öğrenci İşlerine ve Anabilim Dalı Başkanlığına bildirilmesine oybirliği ile karar verildi.

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Tıbbi Biyoloji Ana Bilim Dalı Öğretim Üyesi

Sorumluluğunuzda yapılması tasarlanan "Myleoid Malignansilerde Hematopoetik miRNA ekspresyonlarının Araştırılması" adlı araştırmaya ilişkin 02/11/2016 tarihli ve 2016/344 sayılı Kurul Kararı ile Klinik Araştırmalar Etik Kurulu Karar Formu ekte sunulmuştur.

Bilgilerinizi arz/rica ederim.

Prof. Dr. Bahar TUNÇTAN
Kurul Başkanı

EKLER:

1- Kurulun 02/11/2016 tarihli ve 2016/344 sayılı kararı (1 sayfa)
2- Klinik Araştırmalar Etik Kurulu Karar Formu (3 sayfa)

T.C.
MERSİN ÜNİVERSİTESİ REKTÖRLÜĞÜ
KLİNİK ARAŞTIRMALAR ETİK KURULU

Karar Tarihi	Toplantı Sayısı	Karar Sayısı
02/11/2016	19	344

Mersin Üniversitesi Tıp Fakültesi Temel Tıp Bilimleri Bölümü Tıbbi Biyoloji Ana Bilim Dalı Öğretim Üyesi Yrd. Doç. Dr. Mustafa Ertan AY'ın sorumluluğunda yapılması tasarlanan "Myleoid Malignansilerde Hematopoetik miRNA ekspresyonlarının Araştırılması" adlı araştırma için hazırlanmış olan ve 27/10/2016 tarihinde sunulan Girişimsel Olmayan Klinik Araştırmalar İçin Başvuru Formu ile ilgili belgeler araştırmanın gerekçe, amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiş, araştırmanın yürürlükte olan ilgili yasal düzenlemelere uyularak yürütülmesi ve sonuçlandırılması koşulu ile gerçekleştirilmesinde etik sakınca bulunmadığına toplantıya katılanların oy birliği ile karar verilmiştir.

İmza
Prof. Dr. Bahar TUNÇTAN
Başkan

İmza
Prof. Dr. Selma ÜNAL
Başkan Yardımcısı

İmza
Prof. Dr. Fatma Özlem KANDEMİR
Üye

İmza
Prof. Dr. Olgu HALLIOĞLU KILINÇ
Üye

İmza
Prof. Dr. Murat BOZLU
Üye

İmza
Prof. Dr. Mehmet Sami SERİN
Üye

İmza
Prof. Dr. Bahar TAŞDELEN
Üye

İmza
Prof. Dr. Sabire YURTSEVER
Üye

(Katılamadı)
Doç. Dr. Nimet KARAGÜLLE
Üye

İmza
Doç. Dr. Gamze ÖZÇÜRÜMEZ BİLGİLİ
Üye

İmza
Yrd. Doç Dr. M. Türkan IŞIK ERER
Üye

İmza
Yrd. Doç. Dr. Nalan TİFTİK
Üye

(Katılamadı)
Uzm. Dr. Özge KURMUŞ
Üye

İmza
Hürrem Betül LEVENT ERDAL
Üye

İmza
Lale DAĞLI
Üye

Prof. Dr. Bahar TUNÇTAN
Başkan
ASLI GİBİDİR

KLİNİK ARAŞTIRMALAR ETİK KURULU KARAR FORMU

ARAŞTIRMANIN AÇIK ADI	Myleoid Malignansilerde Hematopoetik miRNA ekspresyonlarının Araştırılması
VARSA ARAŞTIRMANIN PROTOKOL KODU	---

ETİK KURUL BİLGİLERİ	ETİK KURULUN ADI	Mersin Üniversitesi Klinik Araştırmalar Etik Kurulu
	AÇIK ADRESİ:	Mersin Üniversitesi Çiftlikköy Kampüsü Prof. Dr. Uğur ORAL Kültür Merkezi, 33343, Yenişehir/Mersin
	TELEFON	0 324 361 00 01 / 4417
	FAKS	---
	E-POSTA	meukaek@gmail.com

BAŞVURU BİLGİLERİ	KOORDİNATÖR/SORUMLU ARAŞTIRMACI UNVANI/ADI/SOYADI	Yrd.Doç. Dr. Mustafa Ertan AY			
	KOORDİNATÖR/SORUMLU ARAŞTIRMACININ UZMANLIK ALANI	Tıbbi Biyoloji Ana Bilim Dalı			
	KOORDİNATÖR/SORUMLU ARAŞTIRMACININ BULUNDUĞU MERKEZ	Mersin Üniversitesi Tıp Fakültesi Temel Tıp Bilimleri Bölümü			
	VARSA İDARİ SORUMLU UNVANI/ADI/SOYADI	---			
	DESTEKLEYİCİ	---			
	PROJE YÜRÜTÜCÜSÜ UNVANI/ADI/SOYADI (TÜBİTAK vb. gibi kaynaklardan destek alanlar için)	---			
	DESTEKLEYİCİNİN YASAL TEMSİLCİSİ	---			
	ARAŞTIRMANIN FAZI VE TÜRÜ	FAZ 1	☐		
		FAZ 2	☐		
		FAZ 3	☐		
		FAZ 4	☐		
		Gözlemsel ilaç çalışması	☐		
		Tıbbi cihaz klinik araştırması	☐		
		İn vitro tıbbi tanı cihazları ile yapılan performans değerlendirme çalışmaları	☐		
		İlaç dışı klinik araştırma	☐		
		Diğer ise belirtiniz–Rutin muayene, tetkik, tahlil ve tedavi işlemleri sırasında elde edilmiş materyaller ile yapılacak araştırma -Gen tedavisi klinik araştırmaları dışında kalan ve tanımlamaya yönelik olarak genetik materyal ile yapılacak araştırma			
	ARAŞTIRMAYA KATILAN MERKEZLER	TEK MERKEZ ☒	ÇOK MERKEZLİ ☐	ULUSAL ☐	ULUSLARARASI ☐

Prof. Dr. Bahar TUNÇTAN
Etik Kurul Başkanı
İmza:

Not: Etik kurul başkanı, imzasının yer almadığı her sayfaya imza atmalıdır.

KLİNİK ARAŞTIRMALAR ETİK KURULU KARAR FORMU

ARAŞTIRMANIN AÇIK ADI	Myleoid Malignansilerde Hematopoetik miRNA ekspresyonlarının Araştırılması
VARSA ARAŞTIRMANIN PROTOKOL KODU	---

	Belge Adı	Tarihi	Versiyon Numarası	Dili
DEĞERLENDİRİLEN BELGELER	ARAŞTIRMA PROTOKOLÜ			Türkçe ☐ İngilizce ☐ Diğer ☐
	BİLGİLENDİRİLMİŞ GÖNÜLLÜ OLUR FORMU (ERİŞKİN HASTALAR İÇİN)	27/10/2016	---	Türkçe ☒ İngilizce ☐ Diğer ☐
	OLGU RAPOR FORMU			Türkçe ☐ İngilizce ☐ Diğer ☐
	ARAŞTIRMA BROŞÜRÜ			Türkçe ☐ İngilizce ☐ Diğer ☐

	Belge Adı		Açıklama
DEĞERLENDİRİLEN DİĞER BELGELER	SİGORTA	☐	
	ARAŞTIRMA BÜTÇESİ	☒	
	BİYOLOJİK MATERYEL TRANSFER FORMU	☐	
	İLAN	☐	
	YILLIK BİLDİRİM	☐	
	SONUÇ RAPORU	☐	
	GÜVENLİLİK BİLDİRİMLERİ	☐	
	DİĞER: GİRİŞİMSEL OLMAYAN KLİNİK ARAŞTIRMALAR İÇİN BAŞVURU FORMU	☒	
	ARAŞTIRMACILARIN ÖZ GEÇMİŞİ	☒	
	3 ADET LİTERATÜR	☒	
	Diğer	☒	-Çalışmanın Tez Olduğuna İlişkin Kurul Kararı, 18.07.2016
KARAR BİLGİLERİ	**Karar No:** 2016/344	**Tarih:** 02/11/2016	
	Yukarıda bilgileri verilen başvuru dosyası ile ilgili belgeler araştırmanın/çalışmanın gerekçe, amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiş ve uygun bulunmuş olup araştırmanın/çalışmanın başvuru dosyasında belirtilen merkezlerde gerçekleştirilmesinde etik ve bilimsel sakınca bulunmadığına toplantıya katılan etik kurul üye tam sayısının salt çoğunluğu ile karar verilmiştir. İlaç ve Biyolojik Ürünlerin Klinik Araştırmaları Hakkında Yönetmelik kapsamında yer alan araştırmalar/çalışmalar için Türkiye İlaç ve Tıbbi Cihaz Kurumu'ndan izin alınması gerekmektedir.		

KLİNİK ARAŞTIRMALAR ETİK KURULU	
ETİK KURULUN ÇALIŞMA ESASI	İlaç ve Biyolojik Ürünlerin Klinik Araştırmaları Hakkında Yönetmelik, Tıbbi Cihaz Klinik Araştırmaları Yönetmeliği, İyi Klinik Uygulamaları Kılavuzu
BAŞKANIN UNVANI / ADI / SOYADI:	Prof. Dr. Bahar TUNÇTAN

Unvanı/Adı/Soyadı	Uzmanlık Alanı	Kurumu	Cinsiyet		Araştırma ile ilişki		Katılım *		İmza
Prof. Dr. Bahar TUNÇTAN	Farmakoloji	MEÜ Eczacılık Fakültesi Meslek Bilimleri Bölümü Farmakoloji ABD	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Prof. Dr. Selma ÜNAL	Çocuk Sağlığı ve Hastalıkları	MEÜ Tıp Fakültesi Dahili Tıp Bilimleri Bölümü Çocuk Sağlığı ve Hastalıkları ABD	E ☐	K ☒	E ☐	H ☐	E ☐	H ☐	
Prof. Dr. Fatma Özlem KANDEMİR	Klinik Mikrobiyoloji ve Enfeksiyon Hastalıkları	MEÜ Tıp Fakültesi Dahili Tıp Bilimleri Bölümü Enfeksiyon Hastalıkları ABD	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	

Prof. Dr. Bahar TUNÇTAN
Etik Kurul Başkanı
İmza:

Not: Etik kurul başkanı, imzasının yer almadığı her sayfaya imza atmalıdır.

KLİNİK ARAŞTIRMALAR ETİK KURULU KARAR FORMU

ARAŞTIRMANIN AÇIK ADI	Myleoid Malignansilerde Hematopoetik miRNA ekspresyonlarının Araştırılması
VARSA ARAŞTIRMANIN PROTOKOL KODU	---

Prof. Dr. Olgu HALLIOĞLU KILINÇ	Çocuk Sağlığı ve Hastalıkları	MEÜ Tıp Fakültesi Dahili Tıp Bilimleri Bölümü Çocuk Sağlığı ve Hastalıkları ABD	E ☐	K ☒	E ☐	H ☑	E ☑	H ☐	
Prof. Dr. Murat BOZLU	Üroloji	MEÜ Tıp Fakültesi Cerrahi Tıp Bilimleri Bölümü Üroloji ABD	E ☒	K ☐	E ☐	H ☑	E ☑	H ☐	
Prof. Dr. Mehmet Sami SERİN	Mikrobiyoloji	MEÜ Eczacılık Fakültesi Farmasötik Mikrobiyoloji ABD	E ☒	K ☐	E ☐	H ☑	E ☑	H ☐	
Prof. Dr. Bahar TAŞDELEN	Biyoistatistik	MEÜ Tıp Fakültesi Temel Tıp Bilimleri Bölümü Biyoistatistik ve Tıbbi Bilişim ABD	E ☐	K ☒	E ☐	H ☑	E ☑	H ☐	
Prof. Dr. Sabire YURTSEVER	İç Hastalıkları Hemşireliği	MEÜ Sağlık Yüksekokulu Hemşirelik Bölümü İç Hastalıkları Hemşireliği ABD	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Doç. Dr. Nimet KARAGÜLLE	Biyomühendislik	MEÜ Mühendislik Fakültesi Kimya Mühendisliği Bölümü	E ☐	K ☒	E ☐	H ☑	E ☐	H ☑	Katılamadı
Doç. Dr. Gamze ÖZÇÜRÜMEZ BİLGİLİ	Psikiyatri	MEÜ Tıp Fakültesi Dahili Tıp Bilimleri Bölümü Psikiyatri ABD	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Yrd. Doç. Dr. M. Türkan IŞIK ERER	Tıp Tarihi ve Etik	MEÜ Sağlık Yüksekokulu Hemşirelik Bölümü Hemşirelik Esasları ABD	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Yrd. Doç. Dr. Nalan TİFTİK	Farmakoloji	MEÜ Tıp Fakültesi Dahili Tıp Bilimleri Bölümü Tıbbi Farmakoloji ABD	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Uzm. Dr. Özge KURMUŞ	Kardiyoloji	Ufuk Üniversitesi Tıp Fakültesi Dahili Tıp Bilimleri Bölümü Kardiyoloji ABD	E ☐	K ☒	E ☐	H ☑	E ☐	H ☑	Katılamadı
Yüksek Şehir Plancısı Hürrem Betül LEVENT ERDAL	Şehir ve Bölge Planlama/Uluslararası Proje Yönetimi	Mersin Mezitli Belediyesi	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Avukat Lale DAĞLI	Hukuk	Serbest	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	

*:Toplantıda Bulunma

Prof. Dr. Bahar TUNÇTAN
Etik Kurul Başkanı
İmza:

Not: Etik kurul başkanı, imzasının yer almadığı her sayfaya imza atmalıdır.

Oluşturma : 2015-06-01 08:03:37
Düzenleme : 2016-10-26 14:18:00

Adı ve Soyadı	:	Mustafa Ertan AY
Ünvan	:	Yrd. Doç. Dr.
Birim	:	Tıp Fakültesi / Tıbbi Biyoloji ve Genetik Anabilim Dalı
TC Kimlik No	:	22393888362
Doğum Tarihi	:	01 Ocak 1970
Ev Adres	:	MENDERES MAH. 35411 SK. BEYZADE KONUTLARI SİTESİ B BLOK NO: 7B İÇ KAPI NO: 20 MEZİTLİ / MERSİN
İş Adres	:	MERSİN ÜNİVERSİTESİ TIP FAKÜLTESİ TIBBİ BİYOLOJİ A.D. HASTANE KAMPÜSÜ 33110 YENİŞEHİR/MERSİN
Phone	:	+90-324-3610684 (Telefon) +90-324-3610001 / 1130 (Dahili)
Fax	:	+90-324-3412400
e-Mail	:	ertanay@mersin.edu.tr ertan.ay@deu.edu.tr ertanay35@yahoo.com
URL	:	http://apbs.mersin.edu.tr/ertanay
Researcher ID	:	O-9461-2015
Scopus ID	:	14061719800
Orcid ID	:	0000-0002-6152-7450
Scholar ID	:	
h-index	:	6
Puan	:	7708
Yabancı Dil	:	İngilizce, -, ÜDS, 66,125

Öğrenim Durumu

Derece	Bölüm	Üniversite/Kurum	Yıl
DOKTORA	TIBBİ BİYOLOJİ VE GENETİK	DOKUZ EYLÜL ÜNİVERSİTESİ	2005
YÜKSEK LİSANS	TIBBİ BİYOLOJİ VE GENETİK	DOKUZ EYLÜL ÜNİVERSİTESİ	1994
LİSANS	TIP FAKÜLTESİ TIBBİ BİYOLOJİK BİLİMLER BÖLÜMÜ	DOKUZ EYLÜL ÜNİVERSİTESİ	1991

Tezler

Doktora Tezi

1. Kolorektal kanser gelişiminde, p21, p27, p57 siklin bağımlı kinaz inhibitör genleri (CDKI) ile p53 ve p73 tümör supressör geni ekspersyon düzeyi değişimlerinin belirlenmesi Danışman: Dr. Orhan Terzioğlu, Dokuz Eylül Üniversitesi, Sağlık Bilimleri Enstitüsü, Tıbbi Biyoloji (GENETİK) Anabilim Dalı, İzmir, Türkiye, **2005**.

Yüksek Lisans Tezi

1. Down Sendromunun İn Situ Hibridizasyon Yöntemi İle Gösterilmesi Danışman: Dr. Orhan Terzioğlu, Dokuz Eylül Üniversitesi, Sağlık Bilimleri Enstitüsü, Tıbbi Biyoloji (GENETİK) Anabilim Dalı, İzmir, Türkiye, **1995**.

Akademik Görevler

Görev Ünvanı	Üniversite/Kurum	Yıl
YARDIMCI DOÇENT DOKTOR	MERSİN ÜNİVERSİTESİ TIP FAKÜLTESİ TIBBİ BİYOLOJİ ANABİLİM DALI	2006-10-02 - Devam ediyor
AKADEMİK UZMAN	DOKUZ EYLÜL ÜNİVERSİTESİ TIP FAKÜLTESİ TIBBİ BİYOLOJİ VE GENETİK ANABİLİM DALI	1992-03-20 - 2006-10-02

Yayınlar 5590

Uluslararası - SCI/SCI-Expanded kategorisine giren 5310

2016

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10.5455/bcp.20151016123639

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17. Buber, A.; Basay, B.; Basay, O.; Ozturk, O.; Alacam, H.; Yazici, K.; Bacanli, A.; Ay, M.; Erdal, M. Synapsin Polymorphisms Could Be Correlated with Stroop Simple Reaction Time Scores. *AMERİCAN JOURNAL OF MOLECULAR BİOLOGY*, **2016**, *06*, 25-32. 100 + 0
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16. Dasdag, S.; Akdag, M.; Erdal, M.; Erdal, N.; Ay, O.; Ay, M.; Yilmaz, S.; Tasdelen, B.; Yegin, K. Effects of 2.4 GHz radiofrequency radiation emitted from Wi-Fi equipment on microRNA expression in brain tissue. *INTERNATIONAL JOURNAL OF RADIATION BIOLOGY*, **2015**, *91*, 555-561. 200 + 0
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https://dx.doi.org/10.1089/gtmb.2014.0262

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2014

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2012

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2011

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5. Herken, H.; Erdal, E.; Sengul, C.; Yucel, E.; Cakaloz, B.; Kenar, A.; Ay, E.; Ergundu, T. Attention deficit hyperactivity disorder associaton with synaptosomal-associated protein (snap-25) polymorphisms. *KLINIK PSIKOFARMAKOLOJI BULTENI-BULLETIN OF CLINICAL PSYCHOPHARMACOLOGY*, **2009**, *19*, S142-S143. 0 + 0

2007

4. Ay, M.; Ay, I.; Soylemez, F.; Erdal, M. Deletion of 8p: a case of a child with multiple congenital anomalies. *CHROMOSOME RESEARCH*, **2007**, *15*, 62-62. 950 + 0

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Dönem	D.Kodu	Ders	Fakülte/Yüksekokul/Enstitü	Bölüm/Program	T-U-K
Güz	BİO 616	Hücresel Kontrol ve Sinyal İ	Sağlık Bilimleri Enstitüsü	Tıbbi Biyoloji Doktora Progra...	3-0-3
Güz	BİO 801	Uzmanlık Alan Dersi - I	Sağlık Bilimleri Enstitüsü	Tıbbi Biyoloji Anabilim Dalı	4-0-4
Güz	BİO 802	Uzmanlık Alan Dersi - II	Sağlık Bilimleri Enstitüsü	Tıbbi Biyoloji Anabilim Dalı	4-0-4
Güz	SEM YL	Seminer	Sağlık Bilimleri Enstitüsü	Sağlık Bilimleri Enstitüsü	0-0-0

2015-2016 (9 Ders)

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Güz		KALITSAL METABOLİK HASTALIKLA...	Sağlık Bilimleri Enstitüsü	TIBBİ BİYOLOJİ	2-2-3
Güz		MOLEKÜLER İMMÜNOLOJİ	Sağlık Bilimleri Enstitüsü	TIBBİ BİYOLOJİ	2-0-2
Bahar	BİO 802	Uzmanlık Alan Dersi - II	Sağlık Bilimleri Enstitüsü	Tıbbi Biyoloji Anabilim Dalı	4-0-4
Bahar	BİO 801	Uzmanlık Alan Dersi - I	Sağlık Bilimleri Enstitüsü	Tıbbi Biyoloji Anabilim Dalı	4-0-4
Bahar	BİO 506	Sitogenetik	Sağlık Bilimleri Enstitüsü	Tıbbi Biyoloji Yüksek Lisans...	2-2-3
Bahar	BİO 510	Kanserin Moleküler Biyolojisi	Sağlık Bilimleri Enstitüsü	Tıbbi Biyoloji Yüksek Lisans...	2-0-2
Bahar	BİO 619	İleri Sitogenetik	Sağlık Bilimleri Enstitüsü	Tıbbi Biyoloji Doktora Progra...	2-2-3
Bahar	TEZ	Tez	Sağlık Bilimleri Enstitüsü	Sağlık Bilimleri Enstitüsü	0-0-0
Bahar	SEM YL	Seminer	Sağlık Bilimleri Enstitüsü	Sağlık Bilimleri Enstitüsü	0-0-0

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2013-2014 (2 Ders) 300

2012-2013 (1 Ders) 150

2011-2012 (1 Ders) 150

Akademik Özgeçmiş | Mersin Üniversitesi

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Adı ve Soyadı	:	Cemile ERSÖZ
Ünvan	:	Yüksek Lisans Öğrencisi
Birim	:	
TC Kimlik No	:	24946560084
Doğum Tarihi	:	17 Aralık 1988
Ev Adres	:	MENDERES MAH. 35419 SK. 23/10 MEZİTLİ/MERSİN
İş Adres	:	MERSİN ÜNİVERSİTESİ SAĞLIK BİLİMLERİ ENSTİTÜSÜ
Phone	:	+90-- (Telefon) +90-324-3610001 / (Dahili)
Fax	:	+90--
e-Mail	:	ylbio201507@mersin.edu.tr ylbio201507@mersin.edu.tr
URL	:	http://apbs.mersin.edu.tr/ylbio201507
Researcher ID	:	
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