MicroRNAs in Ewing's Sarcoma

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1 Introduction

Development of cancer requires genetic events such as chromosomal translocations and deletions as well as point mutations that affect cell growth, survival and proliferation. In some types of cancer, genetic alterations are believed to be insufficient to induce malignant transformation and epigenetic changes are invoked to play a key role in the establishment of the full malignant phenotype. More recently, microRNA (miRNA) deregulation has been shown to be involved in the development of most, if not all, cancer subtypes (Croce, 2009).

MiRNAs play a pivotal role in regulation of key processes such as development, differentiation and apoptosis. MiRNAs are 19 to 22 nucleotides long and highly conserved across species. MiRNA genes represent approximately 1% of the genome and are commonly transcribed by RNA polymerase II in a long pri-miRNA which is then processed in the nucleus by the Drosha/DGCR8 multiprotein complex to form a 70-100nt long pre-miRNA. Pre-miRNA is exported from the nucleus to the cytoplasm by Exportin-5 where an additional processing step mediated by Dicer generates a miRNA duplex of approximately 22nt. The MiRNA duplex is separated and the guide strand loaded onto the RNA-induced silencing complex (RISC) whereas the carrier strand is degraded. In the RISC mature miRNAs are able to regulate gene expression at the post-transcriptional level by binding the 3' untranslated region (UTR) of messengerRNA (mRNA) leading to mRNA degradation or inhibition of translation, depending on the level of complementarity (figure 1). A single miRNA can target different mRNAs and it is believed that approximately 30% of the genes are subject to post-transcriptional control by miRNAs (Iorio, 2009). Generally, members of the same miRNA family have identical targets because they share a common seed sequence of 7 or 8 nucleotides which interacts with mRNA (Bartel, 2009).

Expression of miRNAs can be altered by several mechanisms. MiRNA loci deletions, amplifications or mutations have been described in various malignancies (Calin, 2002) (Raveche, 2007). In addition, recent publications describe broad miRNA repression or block of maturation in cancer that may contribute to enhance tumorigenesis (Kumar, 2007). Genes encoding proteins involved in the maturation and processing of miRNAs can also be mutated or down-regulated. Indeed, Exportin-5 which transports pre-miRNA from the nucleus to the cytoplasm and the RISC-subunit TARBP2 can both be mutated (Melo, 2009 and 2010). More

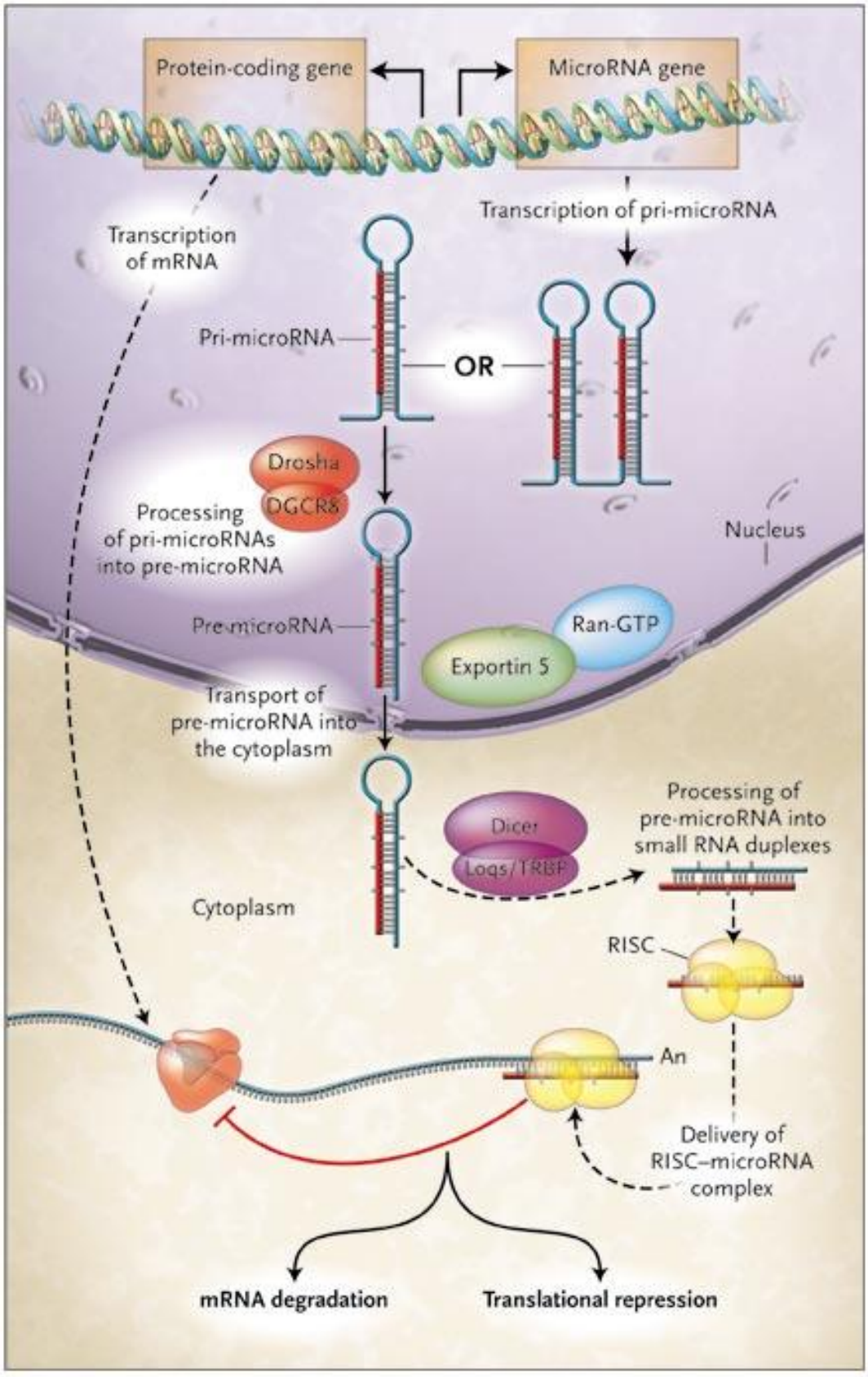

Figure 1 : MicroRNA biogenesis and post-transcriptional regulation.

Chen, NEJM, 2005

recently, it has been shown that several types of cancer can be treated by exogenous administration of specific miRNAs in mouse xenotransplant models (Garzon, 2010). Taken together, these observations indicate that miRNAs are strongly implicated in tumorigenesis and that they are a potentially valuable target for therapeutic intervention.

Recent work from our laboratory has identified several miRNAs that play a role in Ewing's sarcoma and shown that repression of miRNA-145 is implicated in the emergence of cancer stem cells (CSC) and that let-7a down-regulation contributes to Ewing's sarcoma pathogenesis (Riggi, 2010) (De Vito, 2011). Ewing's sarcoma family tumors (ESFT) are the second most common bone malignancy in young adults and children. ESFT are characterized by unique chromosomal translocations that in 85% of cases lead to the expression of the EWS-FLI1 fusion protein t(11;22). EWS-FLI1 acts as an aberrant transcription factor that can both induce and repress target genes. Interestingly, expression of EWS-FLI1 in human mesenchymal stem cells (MSCs), the most likely cell of origin of Ewing's sarcoma, reproduces the ESFT phenotype (Riggi, 2010).

To identify miRNAs that may play a role in ESFT pathogenesis our laboratory performed miRNA profiling of MSCs and different ESFT cell lines. The expression profile revealed a repression of the entire let-7 family (figure 2), miRNA-100, miRNA-125b and miRNA-31. Interestingly, miRNA-100, miRNA 125b and miRNA let-7a belong to the same intergenic cluster and are transcribed as a single pri-miRNA in Drosophila. In contrast, the oncogenic miRNA 17-92 cluster, miRNA-106a and miRNA-106b are induced in ESFT (De Vito, 2011). The let-7 family is of particular interest because it is often deregulated in various malignancies and has been shown to contribute to the emergence of CSC in breast cancer (Yu, 2007). Low levels of let-7 are associated with poor prognosis and tumor aggressiveness (Büssing, 2008). Let-7 is implicated in the post-transcriptional regulation of several oncogenes including c-MYC, k-RAS or HMGA2 (Roush, 2008).

Let-7 family members are regulated by Lin28 and its isoform Lin28B which block their maturation by binding to their terminal loop and preventing their processing by Drosha/DGCR8. Moreover, Lin28/Lin28B are able to recruit a TUTase that uridylates pre-let-7 leading to its degradation (Heo, 2008), (Viswanathan, 2010), (figure 3). Lin28 is a highly conserved RNA binding protein that was first identified in C. elegans where it plays a pivotal

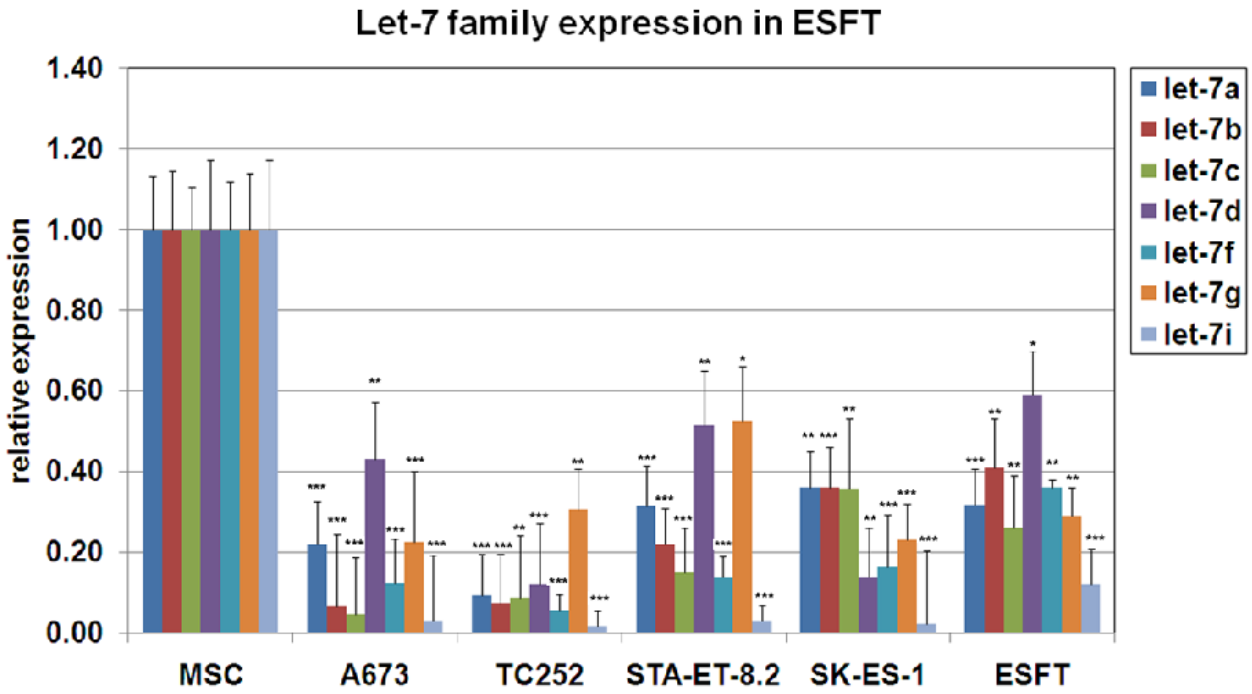

Figure 2

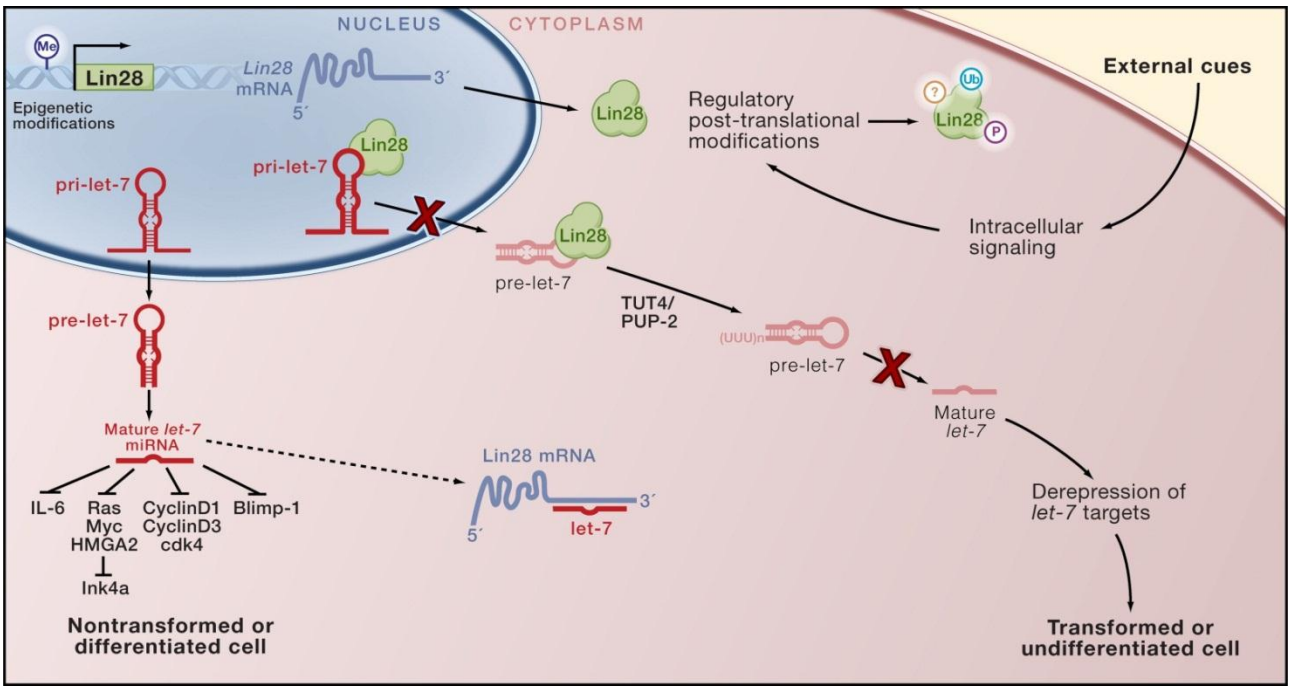

Figure 3

Figure 2 : Relative expression level of let-7 in primary ESFT and different ESFT cell lines reveals a global reduction of all the let-7. De Vito et al., Plos One, 2011 ; Figure 3 : In pluripotent or transformed cells Lin28 is induced and affects the let-7 processing. Lin28 is able to bind the pri-let-7 loop, preventing the processing by DGCR8/Pasha. Furthermore, in the presence of Lin28, the pre-let-7 is uridylated by TUT4 and degraded. In differentiated cells, the Lin28 locus is epigenetically not accessible and the let-7 precursors are processed into mature let-7 miRNAs. As a result, main oncogenes are post-transcriptionally down-regulated (Ras, Myc, HMGA2,…) and the pluripotent state is maintained (e.g Blimp-1). Viswanathan, Cell, 2010.

role in developmental timing. Repression of the let-7 family by Lin28 or Lin28B is important in establishing the pluripotent state. Indeed, Lin28 together with OCT4, SOX2 and NANOG is able to reprogram fibroblasts toward induced pluripotent stem cells (Okita, 2008). Lin28 and Lin28B are expressed in many different tumors with a frequency of 15% and are strongly induced in those that are poorly differentiated and aggressive (Viswanathan,2009). We previously observed that Lin28B, but not Lin28, is overexpressed in primary ESFT and ESFT cell lines compared to MSCs (De Vito, 2011). Lin28B also displays let-7 independent functions, including impairment of processing of other miRNAs or stabilizing the mRNA of the cancer stem cell markers CD133 and LGR5 (Wang, 2010).

The aim of this work was to determine whether broad let-7 family repression can be reproduced in MSCs, the candidate cell of origin of ESFT, by stable expression of Lin28B. EWS-FLI1 itself is also able to modulate the expression level of several miRNAs, including let-7a and miRNA-145. Consequently, we also evaluated the let-7 expression profile of MSCs co-expressing Lin28B and EWS-FLI1. In addition, we determined which let-7 regulated oncogenes are affected by enforced expression of Lin28B. Finally, we evaluated the tumorigenic potential of MSCs expressing EWS-FLI1 and Lin28B.

2 Results

2.1 Expression of EWS-FLI1 & Lin28B in human pediatric mesenchymal stem cells (hpMSCs)

To assess the effect of Lin28B and EWS-FLI1 on let-7 and other miRNAs two vectors containing these genes were constructed.

MSCs are highly sensitive to in vitro manipulation and rapidly undergo apoptosis if infected repeatedly. A vector containing an internal ribosomal entry site (IRES) was therefore used because it permits the expression of two genes with a single retroviral infection.

To obtain Lin28B cDNA, RT-PCR from total RNA of primary ESFT was performed. Specific primers were designed that correspond to isoform 1 of Lin28B with the desired restriction sites. (Isoform 2 has no RNA interacting domain). Lin28B cDNA was amplified by polymerase chain reaction (PCR) and the presence and size of the PCR product were verified on an agarose gel (figure 4a). DNA was extracted using a column kit and was subsequently digested with MluI and PmeI restriction enzymes. The pIRES vector was digested using the same restriction enzymes and the insert was ligated to the adjusted vector. The ligated DNA was precipitated and electroporated into bacteria that we selected for ampicillin resistance. Colonies were picked and screened for the presence of Lin28B by PCR, using one primer designed to anneal on the backbone and another to the insert. Thirteen positive colonies were obtained (figure 4b) and two were grown overnight. A mini-prep was performed to recover the DNA and the vector was sequenced to confirm the presence of Lin28B and to exclude mutations. The same steps were then repeated with EWS-FLI1 in order to insert it at the 5' end of the IRES gene. Finally, a colony containing both EWS-FLI1 and Lin28B on the pIRES vector was grown. Preparative amounts of DNA were recovered for retroviral infection. Lin28B was also inserted into a pMSCV vector.

GP2 cells were transfected with a packaging vector and the $pMSCV^{Lin28B}$ or $pIRES^{EWS\text{-}FLI1+Lin28B}$ vectors. The supernatant containing the viruses was recovered after 72 hours. Two different batches of MSCs were infected with the viruses containing the empty pIRES vector, $pMSCV^{Lin28B}$ or $pIRES^{EWS\text{-}FLI1\ +\ Lin28B}$. Cells were recovered after selection for puromycin resistance and culture in knock out (KO) medium, according to the reprogramming protocol. KO medium is composed of 20% KO Serum Replacement, 10ng/mL PDGF-BB, 1% penicillin-

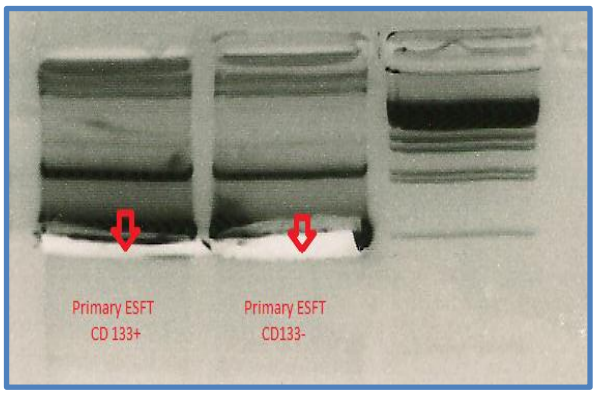

Figure 4a

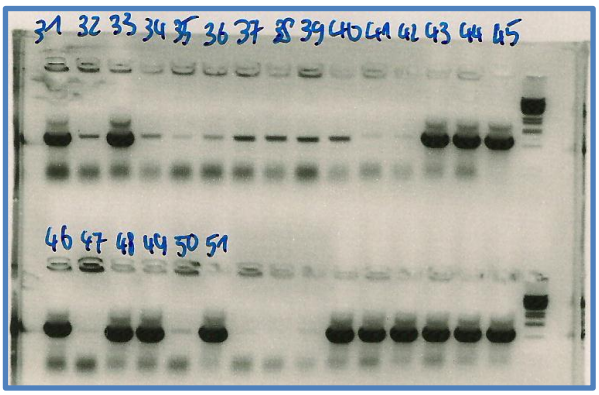

Figure 4b

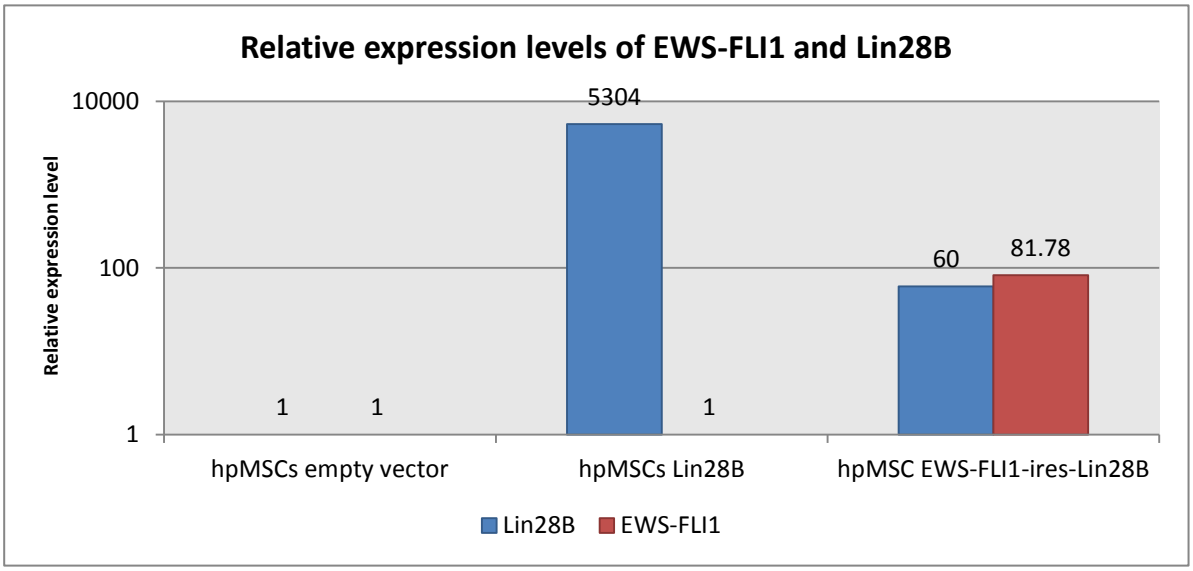

Figure 4c

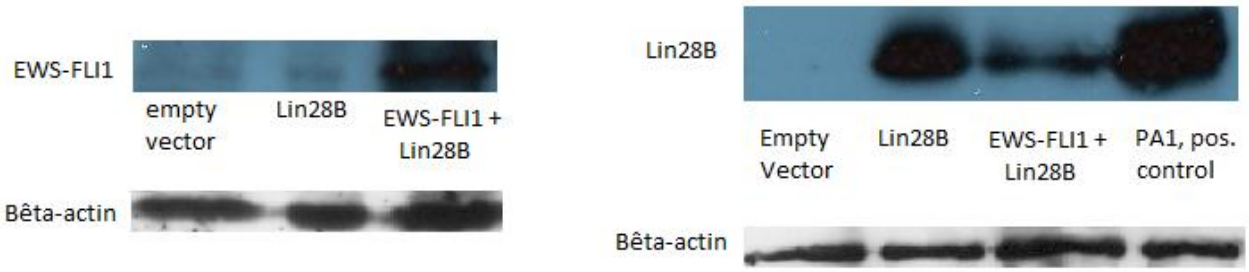

Figure 4d

Figure 4a : PCR of Lin28B from primary ESFT cDNA of CD133+ and CD133- fractions. The bands are cut on the picture (arrow). The primers used contain the MluI and PmeI restriction sites. We observe many aspecific bands. Figure 4b : Combs 41 to 60 : PCR screening of Lin28B in the pIRES vector. The forward primer anneals on the vector, the reverse on Lin28B. Figure 4c : Relative expression level tested by qPCR of EWS-FLI1 and Lin28B in mesenchymal stem cells transfected with either empty vector, Lin28B or EWS-FLI1 and Lin28B. This experiment confirms the integrity of my vectors at the transcriptional level. Figure 4d : Western blot of EWS-FLI1 and Lin28 in order to check the integrity of our vectors at the post-transcriptional level. Lin28B is less expressed in the IRES vector, consistent with the results of the qPCR. PA1 is a cell line who highly expresses Lin28B and is commonly used as a positive antibody control for Lin28B.

streptomycin, and 10ng/mL human recombinant leukemia inhibitory factor (LIF) and increases the reprograming potential of MSCs expressing EWS-FLI1 (See material and methods and Riggi 2010). Expression of Lin28B and EWS-FLI1 was verified by qPCR and western blot analysis (figures 4c and 4d). Total RNA and proteins were extracted and used for subsequent experiments.

2.2 Lin28B impairs expression of let-7 and miRNA-143 in MSCs

The expression level of the different let-7 miRNAs was assessed by qPCR using specific primers. We observed that MSC^{Lin28B} and $MSC^{EWS\text{-}FLI1+Lin28B}$ display a two fold reduction of mature let-7 expression compared to $MSC^{empty\text{-}vector}$ (figure 5). Interestingly, this expression profile is similar to the one observed in primary ESFT and ESFT cell lines. Globally, the let-7 expression level is slightly lower in MSC^{Lin28B} than in $MSC^{EWS\text{-}FLI1+Lin28B}$ probably due to the higher expression levels of Lin28B in the former.

Members of our laboratory recently showed that EWS-FLI1 itself binds the promoter of let-7a thereby repressing its expression (De Vito, 2011). In addition, when EWS-FLI1 is expressed in MSCs the expression of let-7a is significantly reduced whereas the expression of the other miRNAs of the let-7 family remains unaffected (data not shown).

We observe that the expression level of let-7a is significantly lower in MSC expressing both Lin28B and EWS-FLI1. This suggests that in ESFT let-7a is subject to a double repressive regulatory mechanism at the transcriptional as well as the post-transcriptional level, as a result of direct binding of EWS-FLI-1 to its promoter and Lin28B mediated maturation blockade, respectively.

It has been reported that Lin28B also displays let-7 independent functions, such as impairment of the expression of miRNA-107, miRNA-143 and miRNA-200c (Heo, 2009). Our laboratory recently uncovered the importance of mir-143 in Ewing's Sarcoma cancer stem cell maintenance and observed that exogenous administration of mir-143 inhibits ESFT growth in vivo (Unpublished data). Consequently, we assessed the miRNA-143 expression level in MSC^{Lin28B} and $MSC^{EWS\text{-}FLI1\ +\ Lin28B}$. We observed, respectively, a 1.59 and 2.2 fold reduction of the miRNA-143 expression level compared to $MSC^{empty\text{-}vector}$ (figure 5).

Taken together, these results suggest that the overexpression of Lin28B might contribute to ESFT pathogenesis by downregulating two miRNAs that play a role in this malignancy, namely miRNA-let-7a and miRNA-143.

2.3 The let-7 targets IMP-1 and HMGA2 are released in MSC^{Lin28B} and $MSC^{EWS\text{-}FLI1\text{-}Lin28B}$

To evaluate whether Lin28B was able to modulate the expression of let-7 oncogenic targets, qPCR of k-RAS, HMGA2 and IMP-1 was performed on the RNA of $hpMSC^{Lin28B}$ and $hpMSC^{EWS\text{-}FLI1 + Lin28B}$. We observed that HMGA2 and IMP-1 were induced in $hpMSC^{Lin28B}$ and $hpMSC^{EWS\text{-}FLI1 + Lin28B}$ whereas the expression level of k-RAS remained stable (figure 6a). These observations were confirmed by western blot analysis (figure 6b). HMGA2 is a DNA binding protein that may act as a transcriptional regulator. Interestingly, HMGA2 expression has been associated with mesenchymal tumors, including lipomas, leiomyomas and liposarcomas (Henrikson, 2010).

Recent work from our laboratory has shown that EWS-FLI1 directly targets the let-7a promoter thereby releasing the HMGA2 oncogene, which affects tumor growth of ESFT in vivo. Indeed, overexpression of let-7a and repression of HMGA2 both block ESFT cell tumorigenecity. Exogenous administration of let-7a decreased HMGA2 expression levels and reduced ESFT growth in vivo. MSCs expressing both EWS-FLI1 and Lin28B display a higher expression level of HMGA2 than the MSCs expressing only Lin28B, respectively 3.32 and 1.56 fold relative to $MSC^{empty\text{-}vector}$ (Figure 6a). This could be explained by the lower let-7a expression levels observed in $MSC^{EWS\text{-}FLI1 + Lin28B}$ that reflect the cumulative effect of Lin28B mediated let-7a repression and direct inhibition of the let-7a promoter by EWS-FLI1.

IMP-1, also known as insulin growth factor 2 binding protein 1 (IGF2BP1) displayed a 2.2 fold induction in $hpMSC^{Lin28B}$ and a 2.5 fold induction in $hpMSC^{EWS\text{-}FLI1 + Lin28B}$. The specific role that IMP-1 might play in ESFT remains elusive, and not much is known about its role in the pathogenesis of cancer. Hence, very recent data uncovered that low let-7 levels observed in ovarian cancer upregulate IMP-1 which in turn contributes to enhance resistance to chemotherapy with taxanes (Boyerinas, 2011).

The expression level of K-RAS remained stable in hpMSC^{Lin28B} and hpMSC$^{EWS\text{-}FLI1+Lin28B}$ compared to hpMSC$^{empty\ vector}$. The constitutive expression level of k-RAS is low in MSCs and let-7 down-regulation seems insufficient to augment it. Interestingly, in previous experiments we observed that enforced expression of let-7a in ESFT cell lines affected significantly the expression level of HMGA2, IMP-1 and Lin28B but not k-RAS (Unpublished data).

In summary, our results suggest that expressing both EWS-FLI1 and Lin28B in hpMSCs is able to significantly upregulate the let-7 targets HMGA2 and IMP-1 whereas the expression of k-RAS remains unaffected.

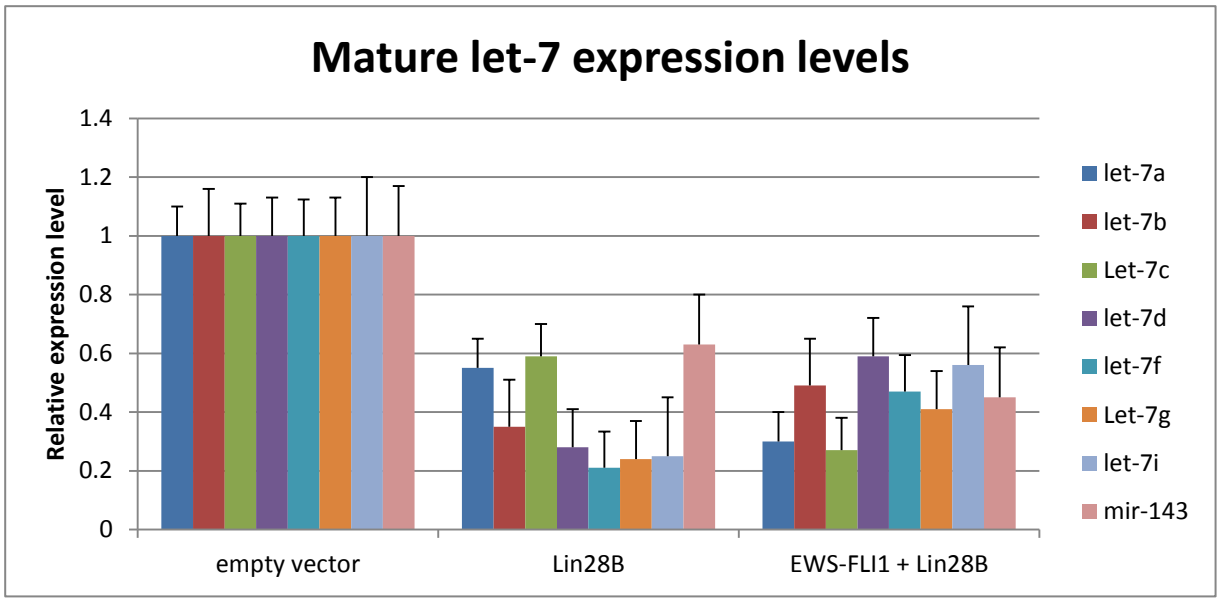

Figure 5

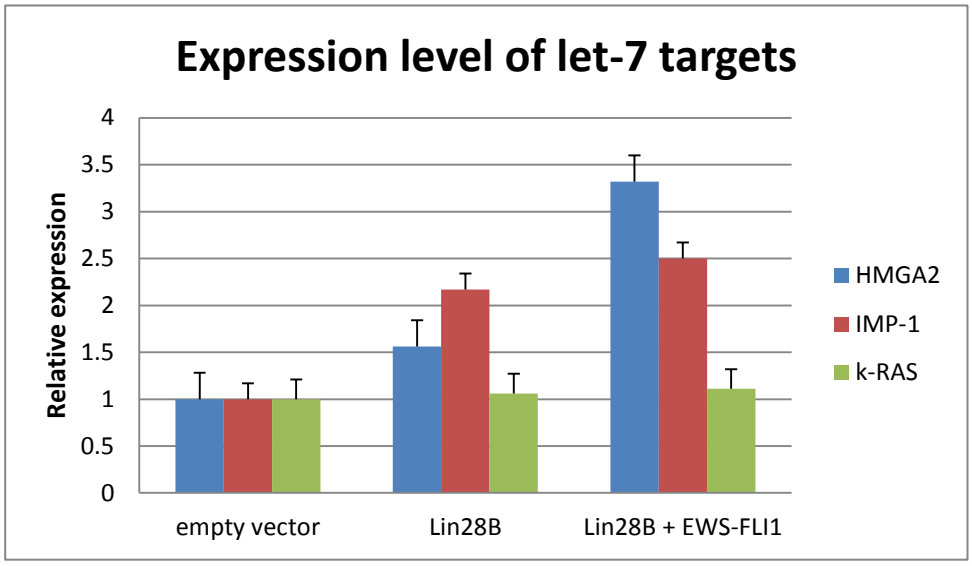

Figure 6a

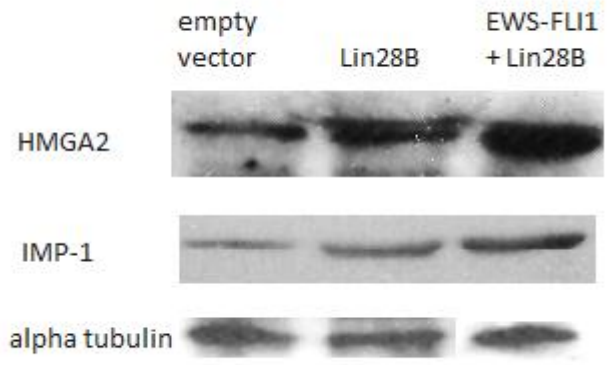

Figure 6b

Figure 5 : expression level of the let-7 family in $MSC^{empty\text{-}vector}$ MSC^{Lin28B} and $MSC^{EWS\text{-}FLI1+Lin28B}$; Figure 6a : Relative expression levels of the let-7 target genes HMGA2, IMP-1 and k-RAS in $MSC^{empty\text{-}vector}$ MSC^{Lin28B} $MSC^{EWS\text{-}FLI1+Lin28B}$; Figure 6b : Western blot analysis of HMGA2 and IMP-1 in $MSC^{empty\text{-}vector}$ MSC^{Lin28B} and $MSC^{EWS\text{-}FLI1+Lin28B}$

2.4 Soft agar colony formation assay of $MSC^{EWS\text{-}FLI1+Lin28B}$

To determine whether MSCs co-expressing EWS-FLI1 and Lin28B had tumorigenic potential we performed a soft agar colony formation assay. Growth in agar is a convenient method to evaluate to ability of cell anchorage independent growth, which is a characteristic of transformed cells. It has been demonstrated that impaired miRNA processing enhances cellular transformation. Low let-7 levels increase the soft agar colony formation capacity and transformation of cells in vivo. Indeed, NIH/3T3 cells overexpressing Lin28B formed colonies in soft agar and tumors after injection in nude mice (Kumar, 2007). In previous experiments we observed that $MSC^{EWS\text{-}FLI1}$ do not grow in soft agar despite their strong genetic and phenotypic resemblance to primary ESFT cells (Riggi, 2010).

$MSC^{EWS\text{-}FLI1+Lin28B}$, MSC^{Lin28B} and the A673 Ewing's sarcoma cell line were plated on agar for 6 weeks. A673 were used as positive control (Figure 7a). We observed no significant growth in of $MSC^{EWS\text{-}FLI1+Lin28B}$ (figure 7c) whereas MSC^{Lin28B} displayed strong proliferation in agar (figure 7b). This experiment confirms the strong transforming capacities of Lin28B as observed in NIH/3T3 cells. Among the hypothesis that we considered to explain the absence of growth of $MSC^{EWS\text{-}FLI1+Lin28B}$ in agar we retained the much lower expression of Lin28B compared to MSC^{Lin28B} because of the relative inefficiency of the IRES system. Furthermore, we suspect that we used the wrong antibody for selection. We will perform the experiments again with other vectors that allow high expression levels of both genes. In further experiments we will determine whether we can transform MSCs toward Ewing's sarcoma cells by modulating other members of the miRNA processing machinery.

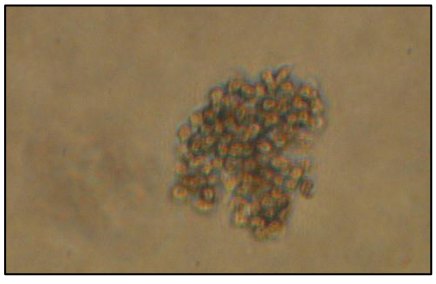

Figure 7a

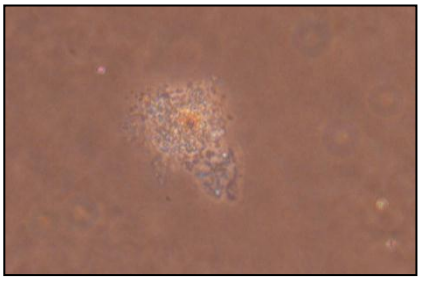

Figure 7b

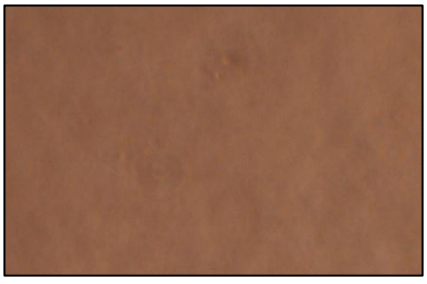

Figure 7c

Figure 7a : Soft agar colony formation assay of A673 Ewing's sarcoma cell lines. ; Figure 7b : Soft agar colony formation assay of MSC^{Lin28b}. ; Figure 7c : Soft agar colony formation assay of $MSC^{EWS\text{-}FLI1 + Lin28B}$

3 Discussion, conclusion and perspectives

3.1 Lin28B impairs the expression of mature let-7 and miRNA-143

Enforced expression of Lin28B and EWS-FLI1 in the cell of origin of ESFT reproduces the let-7 expression profile observed in primary ESFT and in ESFT cell lines. Interestingly, the let-7 family is regulated by multiple oncogenes, all leading to let-7 down regulation. As discussed above, let-7a seems to be repressed in at least two ways in ESFT: by Lin28B blockade of maturation and by direct repression of the let-7a promoter by EWS-FLI1. C-MYC which is induced by EWS-FLI1 itself in ESFT has been reported to transcriptionally repress let7-a (Dauphinot, 2001). In addition, it has been shown that c-MYC can induce Lin28B (Chang, 2009). Hence, Lin28B is not induced in $MSC^{EWS\text{-}FLI1}$ and the mechanisms leading to its expression in ESFT are not understood yet. Direct repression of let-7a by EWS-FLI1 which in turn upregulates Lin28B seems insufficient to induce its expression in $MSC^{EWS\text{-}FLI1}$. MiRNA-125b, a reported Lin28B target, is strongly repressed in ESFT (McKinsey, 2011). MiRNA-125b belongs to the same cluster as let-7a and it is tempting to speculate that its promoter could be directly repressed by EWS-FLI1 itself. It would be interesting to determine whether EWS-FLI1 binds the mir-125b promoter and how it affects the expression level of Lin28B. To better understand the meaning of Lin28B mediated let-7 repression we could silence the expression of the Lin28B gene in ESFT cell lines using a specific small hairpin RNA (shRNA) and assess how it affects tumor growth, proliferation and differentiation.

It has been reported that Lin28B can also affect the processing of pre-miRNA-107, pre-miRNA-143, and pre-miRNA-200c by recruiting TUT4 which leads to urydilation of the precursor preventing its processing by Dicer (Heo, 2009). Of particular interest is miRNA-143 because our laboratory recently uncovered its central role in ESFT CSC maintenance and showed that exogenous administration of miRNA-143 significantly reduces ESFT tumor growth in vivo (Unpublished data). It would be interesting to compare the expression level of Lin28B and miRNA-143 in the CD133+ and CD133- fraction of primary ESFT to elucidate more fully the role that Lin28B might play in ESFT CSC maintenance or emergence.

3.2 Let-7 repression leads to upregulation of the HMGA2 and IGF2BP1 oncogenes

To understand the significance of Lin28B mediated let-7 repression we analyzed the expression level of let-7 regulated oncogenes, namely HMGA2, IGF2BP1 and k-RAS. HMGA2 is of particular interest because of its association with benign and malignant mesenchymal tumors and its implication in ESFT growth (De Vito, 2011). It could be of interest to further study the role of HMGA2 in the promotion of stemness and CSC maintenance. Indeed, recent data revealed that HMGA2 regulates neural stem cell self-renewal by controlling the INK4a locus (Nishino, 2008). Our observations suggest that the cumulative repression of let-7a by Lin28B and EWS-FLI1 in turn leads to a robust induction of HMGA2 in $MSC^{EWS\text{-}FLI1 + Lin28B}$. Furthermore, because of the predicted EWS-FLI1 binding sites in the HMGA2 promoter we could determine whether EWS-FLI1 is able to induce HMGA2 directly.

IMP-1 also seems to be affected by Lin28B-mediated let-7 repression in MSC^{Lin28B} and $MSC^{EWS\text{-}FLI1+Lin28B}$. The specific role that IMP-1 might play in ESFT pathogenesis has not been studied. However, in ovarian cancer, low let-7 and high IMP-1 levels are frequently observed and associated with poor prognosis (Köbel, 2007). Ovarian cancers are frequently treated with chemotherapy regimens that combine carboplatin and taxanes. Unfortunately, resistance to taxanes is often observed. Recent work showed that low let-7 levels observed in ovarian cancer upregulate IMP-1 which stabilizes the Multi-Drug-Resistance-1 protein and in turn contributes to taxane treatment resistance (Boyerinas 2011). Another known function of IMP-1 is to directly bind and stabilize c-MYC mRNA leading to an increase in c-MYC protein abundance (Köbel, 2007). IMP-1 is expressed only in a small subset of tissues, predominantly of fetal origin. Interestingly a study has shown that 73% of malignant mesenchymal tumors express IMP-1 and that 14 out of 14 Ewing's Sarcomas tested were positive for IMP-1 (Ioannidis, 2001). This suggests that IMP-1 may play a role in mesenchymal tumor pathogenesis and it would be interesting to investigate its putative function in that of ESFT. IMP-1 can bind and stabilize the mRNA of IGF-II (Weidensdorfer, 2009). The IGF signaling pathway is strongly implicated in ESFT growth and survival, but the role of IGF-II in ESFT is uncertain and several ESFT cell lines do not express IGF-II.

3.3 Lin28B : miRNA regulation, pluripotency and CSC generation ?

Our laboratory previously showed that hpMSC$^{EWS-FLI1}$ display a transcriptome that is highly similar to that of ESFT and uncovered the mechanisms by which EWS-FLI1 initiates MSC reprogramming toward Ewing sarcoma cancer stem cells. EWS-FLI1 is able to modulate the expression of several embryonic stem cell genes, in part through repression of mir-145. Human pediatric MSC$^{EWS-FLI1}$ grown under serum-free conditions display a strong induction of SOX2, OCT4 and NANOG which are implicated in ESC maintenance and reprogramming. Strikingly, the expression of EWS-FLI1 in hpMSC is able to generate a subpopulation of CD133+ cells, which has been shown to resemble Ewing Sarcoma CSC. The CD133+ fraction of hpMSC$^{EWS-FLI1}$ has sphere forming ability in vitro but no tumorigenic properties in vivo (Suva, 2009) (Riggi, 2010).

Let-7 family microRNAs are strongly repressed in ESFT and let-7 inhibition by Lin28/Lin28B has been associated with malignant transformation in various types of cancer. Moreover, Lin28 plays a role in cancer stem cell maintenance and can stabilize the mRNA of the stem cell markers CD133/Prominin1 and LGR5 through let-7 independent mechanisms in colon cancer (Yang, 2010) (King, 2011). The mechanism by which Lin28B is overexpressed in ESFT is unclear and EWS-FLI1 seems unable to induce its expression in hpMSC. Based on our observations which suggest that Lin28B might globally reduce mature let-7 expression and in turn upregulate oncogenes, we stably co-expressed EWS-FLI1 and Lin28B in hpMSC. The aim was to assess, by soft agar colony formation assay, whether upregulating Lin28B can lead to cellular transformation. Lin28B is of particular interest in this context because it seems to play a role in CSC constitution and maintenance by affecting miRNA maturation in breast cancer. (Yang, 2010) Co-expressing EWS-FLI1 and Lin28B was not able to transform primary hpMSCs toward Ewing's sarcoma cells with malignant properties. However, when we expressed Lin28B at high levels in MSCs we observed growth in soft agar, confirming the transforming capacities of Lin28B. Despite absence of growth in soft agar of MSC expressing both EWS-FLI1 and Lin28B, most likely because of inadequate expression of Lin28B, the observation that Lin28B alone can transform MSC provides a novel and valuable clue as to the molecular components required for MSC transformation to ESFT.

In future experiments we will determine whether we can transform MSCs toward Ewing's sarcoma cells by modulating the expression level of other members of the miRNA processing machinery such as Dicer, DGCR8 or TARBP2. We will also determine whether the global ESFT miRNA expression profile can be reproduced by affecting a specific processing step. Finally, we will evaluate whether we can block ESFT growth in mice by exogenous administration of specific miRNAs or by pharmacologically targeting components of the processing machinery.

4 Material and methods

Cell culture

hpMSCs were obtained from femoral head bone marrow of two pediatric patients undergoing limb axis correction surgery. MSCs were cultured at low confluence in IMDM, 10% FCS, and 10ng/ml PDGF-BB (PeProtechEC), and were previously tested for multilineage differentiation into adipocytes, chondrocytes, and osteoblasts.

Cloning and RT-PCR

Lin28B cDNA was amplified from primary ESFT RNA using SuperScript one-step RT-PCR with the platinum Taq kit (Invitrogen). The amplified fragment was digested with BamHI and PmeI and inserted into a pMSCV Puro retroviral vector (BD Biosciences Clontech). Primer sequences for Lin28B were Fwd 5'-CGGGATCCCGGCCACCATGGCCGAAGGCGGGGCTAGCAAAG-3' and Rev 5'-GGGTTTAAACCCTTATGTCTTTTTCCTTTTTTGAACTGAAGGCCCCTTTT-3'. Lin28B and EWS-FLI1 amplified fragments were digested with respectively MluI/PmeI and BglII/HpaI and inserted into a pMSCV-IRES2 retroviral vector (BD Biosciences Clontech). The primer sequences for Lin28B were Fwd 5'-CGACGCGTCGGCCACCATGGCCGAAGGCGGGGCTAGCAAAG-3' and Rev-5'-GGGTTTAAACCCTTATGTCTTTTTCCTTTTTTGAACTGAAGGCCCCTTTT-3'.

Retroviral infection

Expression of Lin28B and EWS-FLI1+Lin28B in hpMSCs was achived using Retroviral Gene Transfer and Expression (BD Biosciences Clontech), according to the manufacturer's recommendations. Expression of the fusion genes and corresponding proteins were tested in all of the batches of cells by RT-PCR and Western blot analysis, respectively, using the mouse anti-V5 antibody (Invitrogen) for EWS-FLI1 and the Lin28B antibody (CellSignaling). Infected cells were selected with 0.75 µg/mL puromycin for 5 days, and the bulk of the resistant cells was used in subsequent experiments.

Western blot

Cell lysis, SDS-PAGE and blotting were performed by standard procedures, and protein bands were detected using a chemoluminescent substrate kit (Pierce) according to the manufacturer's recommendations. Primary mouse anti-V5 epitope (Invitrogen), mouse antiβ-actin (Sigma), Lin28B (CellSignaling) and IMP-1 (CellSignaling). Secondary antibodies were HRP-conjugated goat anti-mouse (Bio-Rad) and goat anti-rabbit immunoglobulin (Dako).

Reprogramming protocol

hpMSCs were cultured in standard FCS-containing medium and infected following the standard retroviral protocol. Forty-eight hours after the retroviral infection, hpMSCs were washed and incubated in KO medium composed of 20% KO Serum Replacement (Invitrogen, GIBCO), 10ng/mL PDGF-BB, 1% penicillin-streptomycin, and 10ng/mL human recombinant leukemia inhibitory factor (LIF) (Millipore; Chemicon).

Real-time quantitative RT-PCR

Total RNA was isolated using Trifast (Peqlab) according to the manufacturer's recommendations. cDNA was obtained using M-MLV reverse transcriptase and RNase H minus (Promega). Typically, 500ng of template total RNA and 250ng of random hexamers were used per reaction. Real-time PCR amplification was performed using a TaqMan Universal PCR mastermix or Power SYBR mix and specific PCR primers in an ABI Prism 7900 instrument (Applied Biosystems). Relative quantification of each target, normalized to an endogenous control Cyclophyllin A, was performed using a comparative Ct method (Applied Biosystems). Probes used included k-RAS. SYBR Green primer sequences for the quantification of Lin28B, EWS-FLI1, HMGA2 and IMP-1 … transcripts were as follows Lin28B Fwd : 5'-GGATTTGGATTCATCTCCATGATAA-3', Lin28B Rev : 5'-GAATTCCACTGGTTCTCCTTCTTTT-3', EWS-FLI1 Fwd : 5'-GTCAACCTCAATCTAGCACAGGG-3',

EWS-FLI1 Rev : 5'-CTGTCGGAGAGCAGCTCCAG-3', HMGA2 Fwd : 5'-GCGCCTCAGAAGAGAGGAC-3', HMGA2 Rev: GTCTTCCCCTGGGTCTCTTAG-3', IMP-1 Fwd 5'-GGCCTGAGAATGAGTG-3', IMP-1 Rev 5'-GAGGGGCAGACAGTGTTG-3'. For microRNA quantification, 30ng of total RNA were amplified using miRCURY LNA Universal RT microRNA PCR kit (Exiqon, DK) according to the manufacturer's recommendations. LNA PCR primers from Exiquon were used for RT-PCR amplification and snord49a was used as endogenous control.

Soft agar colony formation assay

For the base agar 1% agar was melted in microwave and cooled to 40°C in a water bath. 2X KO medium was warmed to 40°C. The two solutions were mixed to give 0.5% agar + 1x KO medium. 1.5 ml was poured into 35mm Petri dishes. Cells were trypsinized, counted (5000 cells/well) and plated on the base agar. For the top agar 0.7% agar was melted and 2X KO medium was warmed to 40°C. 3ml of 2X KO medium was mixed to 3ml 0.7% agar. 1ml of the top agar mix was added to each plate. 2ml medium was added as top layer and then, medium was changed twice a week. Plates were stained with 0.5 ml of 0.005% Crystal Violet for > 1 hour, colonies were counted using a dissecting microscope. The plates were incubated at 37°C for 5 weeks.

N.B this experiment was performed by Aurélie Formey.

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