MicroRNA-155 Targets SMAD2 and Modulates the Response of Macrophages to Transforming Growth Factor-\(\beta\)\(^\text{a,b}\)

Received for publication, May 20, 2010, and in revised form, October 21, 2010 Published, JBC Papers in Press, October 29, 2010, DOI 10.1074/jbc.M110.146852

Fethi Louafi, Rocio T. Martinez-Nunez, and Tilman Sanchez-Elsner

From the JunkRNA Laboratory, Division of Infection, Inflammation and Immunity, University of Southampton School of Medicine, Southampton General Hospital, Southampton SO16 6YD, United Kingdom

Transforming growth factor-beta (TGF-\(\beta\)) is a pleiotropic cytokine with important effects on processes such as fibrosis, angiogenesis, and immunosuppression. Using bioinformatics, we identified SMAD2, one of the modulators of TGF-\(\beta\) signaling, as a predicted target for a microRNA, microRNA-155 (miR-155). MicroRNAs are a class of small non-coding RNAs that have emerged as an important class of gene expression regulators. MiR-155 has been found to be involved in the regulation of the immune response in myeloid cells. Here, we provide direct evidence of binding of miR-155 to a predicted binding site and the ability of miR-155 to repress SMAD2 protein expression. We employed a lentivirally transduced monocyte cell line (THP1–155) containing an inducible miR-155 transgene to show that endogenous levels of SMAD2 protein were decreased after sustained overexpression of miR-155. This decrease in SMAD2 led to a reduction in both TGF-\(\beta\)-induced SMAD-2 phosphorylation and SMAD-2-dependent activation of the expression of the CAGA\(_{12}\)LUC reporter plasmid. Overexpression of miR-155 altered the cellular responses to TGF-\(\beta\) by changing the expression of a set of genes that is involved in inflammation, fibrosis, and angiogenesis. Our study provides firm evidence of a role for miR-155 in directly repressing SMAD2 expression, and our results demonstrate the relevance of one of the two predicted target sites in SMAD2 3'UTR. Altogether, our data uncover an important role for miR-155 in modulating the cellular response to TGF-\(\beta\) with possible implications in several human diseases where homeostasis of TGF-\(\beta\) might be altered.

Transforming growth factor-\(\beta\) (TGF-\(\beta\)) is a pleiotropic cytokine with important effects on a wide range of cells and processes (1). TGF-\(\beta\) has major actions in wound healing/tissue remodeling, being pro-fibrotic, pro-angiogenic, and anti-proliferative (2–4). Importantly, TGF-\(\beta\) can also act as an immunosuppressive and anti-inflammatory cytokine with key roles in inflammation and in some types of cancer (4, 5). For example, some tumors have reduced responsiveness to the anti-proliferative effects of TGF-\(\beta\) leading to a reduction in both TGF-\(\beta\)-induced SMAD2 expression, and our results demonstrate the relevance of one of the two predicted target sites in SMAD2 3'-UTR. Additionally, our data uncover an important role for miR-155 in modulating the cellular response to TGF-\(\beta\) with possible implications in several human diseases where homeostasis of TGF-\(\beta\) might be altered.

It is clearly vital that a major cytokine such as TGF-\(\beta\), which has such a wide range of effects in key physiological processes, must be under tight regulation to control its expression and activity. This regulation should include mechanisms that allow a variety of effects depending on different cellular and tissue contexts (1). In recent years, a new layer of general control of gene expression has been identified and shown to be specifically involved in the TGF-\(\beta\) pathway; that is, microRNAs (miRNAs or miRs)\(^\text{3}\) (9). miRNAs are a class of small non-coding RNAs present in plant and animal cells that play a major role in post transcriptional regulation of protein coding genes (9). MiRs are first transcribed as a long transcript, pri-miRNA, which is subsequently processed into a shorter pre-miRNA in the nucleus. The pre-miR is then exported to the cytoplasm where it is matured into a 21–22-nucleotide-long miR by Dicer protein. MiRs are able to regulate the translation of their target mRNAs by interacting with their 3'-UTR either inhibiting their translation or enhancing their degradation (10, 11). They have recently emerged as critical elements in regulation of gene expression, and variation in their levels of expression is important during normal development, immune regulation, and in disease (12).

There are several studies showing a relationship of TGF-\(\beta\) with a microRNA, miR-155. In human fibroblasts, TGF-\(\beta\) down-regulates the expression of miR-155 (13) while up-regulating the expression of the human angiotensin II type 1 receptor. Interestingly, human angiotensin II type 1 receptor (hAT1R) is a direct target for miR-155, which seems to be then repressing the effect of TGF-\(\beta\) on hAT1R-mediated vasoconstriction. However, underscoring the pleiotropic nature of TGF-\(\beta\), in murine epithelial cells miR-155 is shown to be up-regulated by TGF-\(\beta\) and to be key in the TGF-\(\beta\)-induced epithelial-mesenchymal transition and tight junction dissolution (14).

\(^{\text{a}}\) This work was supported by Medical Research Council Grant G0801984 (to T. S.-E.).
\(^{\text{b}}\) The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S0–S4.

1 Supported by Medical Research Council Grant G0801984.
2 To whom correspondence should be addressed: General Hospital MP 813, Tremona Rd., Southampton SO16 6YD, UK. E-mail: T.Sanchez-Elsner@soton.ac.uk.

3 The abbreviations used are: miRNA or miR, microRNA; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; MMP9, matrix metalloproteinase-9; ICAM1, intercellular adhesion molecule-1, CDS4; IL-4R, IL-4 receptor; qPCR, quantitative PCR.
miR-155 is one of the most studied miRNAs and is a typical multifunctional miRNA (15). It is expressed in monocytes, macrophages, and dendritic cells and has been shown to have an important role in inflammation. Some environmental factors or proinflammatory ligands such as bacterial lipopolysaccharide, polyinosinic-polycytidylic acid, or interferon-β have been shown to induce miR-155 (16, 17). An example of the importance of miR-155 in disease and immunity is seen in patients with the autoimmune disorder rheumatoid arthritis. The levels of miR-155 in monocytes present in synovial fluid of the inflamed joint are much higher than in the peripheral blood monocytes (18). There is also some evidence that miR-155 can act in some situations as an oncomir, as it is up-regulated and seems to be a key factor in the carcinogenic process in neoplastic disease (15, 19).

Employing a bioinformatic prediction (Targetscan (20)), we found a possible direct link between miR-155 and the TGF-β pathway. The 3′-UTR of SMAD2 has two sites at which binding of miR-155 would be predicted, which led us to hypothesize that miR-155 has an important role in modulating TGF-β signaling. Therefore, we have investigated the role of miR-155 in controlling levels/activity of human SMAD2 and have shown that it does indeed affect the TGF-β signaling pathway. We first proved the direct targeting of SMAD2 by miR-155 by employing a fusion construct of the 3′-UTR sequence of SMAD2 coupled to renilla luciferase. Furthermore, we showed that SMAD2 protein levels and phosphorylated SMAD2 were reduced in monocytes overexpressing miR-155. We then proved that this affects the transcriptional activity of a TGF-β-dependent promoter. Thus, our results suggest that miR-155 regulates the ability of myeloid cells to respond to TGF-β, with important effects on fibrosis, angiogenesis, and immunity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—THP-1 and HeLa cell lines were obtained from the ATCC culture collection. THP1–155 cells were cultured in RPMI complete medium, and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum. Recombinant human TGFβ1 was purchased from PeproTech EC Ltd. Doxycycline hyclate was purchased from Sigma.

**Vectors Generated**—For pCDNA3.1.BIC, the genomic region encompassing miR-155 was amplified and cloned into HindIII/XhoI of the pCDNA3.1 multicloning site as previously described (17). pRLTK_WT_3′-UTR_SMAD2 was generated by cloning the 3′-UTR of human SMAD2 into XbaI and NotI sites of the pRLTK vector (Promega). SMAD2 3′-UTR was amplified from genomic DNA by PCR amplification following the protocol established by Ralser et al. (35) using the following primers: 3′-UTR SMAD2 XbaI FOR1 (TCTAGAGTAAGCTTATTACACTAATAGG-TGC) and 3′-UTR SMAD2 REV NotI (GGGCGGGCATG-GTAAACAACAAAAATGCG). pRLTK_MUT1_3′-UTR_SMAD2, pRLTK_MUT2_3′-UTR_SMAD2, and pRLTK_MUT3_3′-UTR_SMAD2 were generated by site-directed mutagenesis on pRLTK_WT_3′-UTR_SMAD2. pRLTK_MUT1_3′-UTR_SMAD2 was generated by site-directed mutagenesis on pRLTK_MUT2_3′-UTR_SMAD2. These vectors were mutated in the putative miR-155 binding sites (detailed in supplemental Fig. S1) using the following primers: 3′-UTR_MUT1_SMAD2_FOR (CTCTGATGGCTCGAGTCTGATTAGATGATGG) and 3′-UTR_MUT1_SMAD2_REV (CCATCATATTGCTTATACAGCAACGTGCAGCCATCGAG) and 3′-UTR_MUT2_SMAD2_FOR (GGTTGGAAGTTTCTTACGTCAGGAGAAAGACCATTGG) and 3′-UTR_MUT2_SMAD2_REV (CTCAATAATGGTCTTCTTCTAGCAGCTAGAAAACGTGC-TCACC). Mutagenesis was done using the QuikChange® site-directed mutagenesis kit (Stratagene) and following the manufacturer’s instructions.

**Transfections**—To generate the THP1–155 cell line, HEK293 T cells were transfected with Superfect (Qiagen) following the manufacturer’s protocol with 5 μg of pLVTHM_BIC (or pLV/ITR_KRAB_Red in the case of generating the repressor lentiviral particles), 3.75 μg of pPA2x, and 1.5 μg of pMD2G. Supernatant from these cells containing lentiviral particles was added to THP-1 cells that were pre-incubated in the presence of 8 μg/ml Polybrene (Sigma) for 30 min at 37°C. Infection was checked 4 days after infection, and positive cells were sorted. All the vectors used in the lentiviral system were kindly provided by Prof. Didier Trono (Ecole Polytechnique Fédérale de Lausanne, Switzerland). For the luciferase promoter assays, we used the CAGA₂₅LUC reporter plasmid kindly provided by Dr. L.M. Botella (Centro de Investigaciones Biologicas, Consejo Superior de Investigaciones Científicas, Spain) (21), which contains the SMAD2 response elements and pRLTK (Promega) as the normalizing vector. THP1–155 cells were transiently transfected using DEAE-dextran mammalian transfection kit (Stratagene) following standard procedures. To assess direct targeting of miR-155, pRLTK, pRLTK_WT_3′-UTR_SMAD2, pRLTK_MUT1_3′-UTR_SMAD2, pRLTK_MUT2_3′-UTR_SMAD2, or pRLTK_MUT3_3′-UTR_SMAD2 were transfected into HeLa cells employing Superfect (Qiagen) following the manufacturer’s instructions. pCDNA3.1.BIC or pCDNA3.1 empty vector was co-transfected. Normalization was achieved with co-transfection of pGL3 (Promega). All dual luciferase assays were measured employing the Dual-Glo kit from Promega. The experiments were performed three times in triplicate. Statistical differences were determined using Student’s t test.

**Transfection of siRNAs and Pre-miRs**—THP1 cells were transfected with 30 nm siRNAs (SMAD2 pre-designed siRNA ID 107873 and custom SMAD2 siRNA (sense GUCCCAUU-GAAAAGACUUAA/antisense UUAAGUUUUCAUGGGA)) with a control siRNA (4390846) and 100 nm pre-miRs (Pre-miR control ID AM17110, pre-miR-155 ID PM12601, and pre-miR-221 ID PM10337) (Applied Biosystems) using Interferin (Polyplus Transfections) according to the manufacturer’s instruction.

**RT and qPCR Analysis**—RNA samples were obtained using the TRizol isolation (Invitrogen) method. Real time PCR using Applied Biosystems TaqMan® MicroRNA assays was used to detect both mature miR-155 and the housekeeping RNU44, which was used as normalizing control. These assays were performed following the manufacturer’s instructions. Briefly, a stem loop primer was used for reverse transcription of 10 ng...
miR-155 Regulates TGF-β Signaling by Targeting SMAD2

of total RNA in two steps (30 min, 16 °C; 30 min, 37 °C) followed by qPCR employing the FAM-TaqMan probe (Applied Biosystems). The FAM-TaqMan probe was used for all real time qPCR detection for our gene expression array (Applied Biosystems).

Western Immunoblotting—Cells were lysed in Nonidet P-40 (1%), 2 mM Pefablock, and 2 μg/ml aprotinin, leupeptin, and pepstatin. 30–50 μg of cell lysates were subjected to SDS-PAGE under reducing conditions and transferred onto an Immobilon polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). Protein detection was performed using the SuperSignal West Pico chemiluminescent system (Pierce). Antibodies employed were β-actin (ab-8227/ABCAM), Smad2 (3122/Cell Signaling Technology), Phospho-Smad2 (3101/Cell Signaling Technology), Smad3 (9523/Cell Signaling Technology), Smad4 (9515/Cell Signaling Technology), and Phospho-Smad3 (9520/Cell Signaling Technology). Quantity One software was used to quantify Smad2 and phospho-Smad2 and β-actin.

Statistical Analysis—Statistical significance was assessed by use of Student’s t test (GraphPad statistical package).

RESULTS

miR-155 Directly Targets Human SMAD2—From a bioinformatics data base (20) miR-155 was predicted to target SMAD2 at two distinct sites at the end of its 3′-UTR. The first site is a 7-mer-1A, located at position 8241–8247, and the second is an 8-mer, located at position 8683–8689. To provide evidence for the first time of a direct link between miR-155 and human SMAD2 and the relevance of these two different seeding regions, we performed a dual luciferase reporter assay in HeLa cells. For this assay, a region of the 3′-UTR of SMAD2 was fused to the renilla luciferase reporter (pRLTK_WT_3′-UTR_SMAD2). This region contains the seeding sequences for miR-155, which allowed us to generate three more constructs in which these seeding regions were mutated. The first construct had only the 7-mer-1A site mutated pRLTK_MUT1_3′-UTR_SMAD2, the second construct had the 8-mer site mutated pRLTK_MUT2_3′-UTR_SMAD2, and the third construct had both sites mutated pRLTK_MUT3_3′-UTR_SMAD2 (Fig. 1). The dual luciferase assay was then done using HeLa cells co-transfected with pGL3 (normalizing control) and either pRLTK (control), pRLTK_WT_3′-UTR_SMAD2 (WT), pRLTK_MUT1_3′-UTR_SMAD2 (MUT1), pRLTK_MUT2_3′-UTR_SMAD2 (MUT2), or pRLTK_MUT3_3′-UTR_SMAD2 (MUT3). These plasmids were co-transfected with pCDNA3.1 (control) or pCDNA3.1-BIC (miR-155). Normalized renilla-luciferase values were represented relative to the control in each case. Data represent the mean value ± S.D. of four different experiments. Statistical significant difference (p ≤ 0.01) is shown as double asterisks.

SMAD2 Protein Levels in Macrophages Are Down-regulated by miR-155—SMAD2 is a key signal transducer and transcription factor in the TGF-β signaling pathway. The finding above, showing direct targeting of SMAD2 by miR-155, indicates that this important pathway can be negatively regulated by miR-155 through targeting of SMAD2. TGF-β is well known for its actions as a potent immunosuppressive and anti-inflammatory cytokine (22). miR-155 has been shown to be linked to a more proinflammatory phenotype in macrophages (16). Hence, we were interested in studying the interaction of miR-155 with the TGF-β signaling pathway in macrophages. We used the monocytic cell line, THP1–155, in which the expression of a lentivirally transduced transgene of miR-155 would allow us to determine the effects of increased levels of miR-155 on SMAD2 protein levels. miR-155 expression was induced over a period of 96 h after doxycycline treatment, and as expected, it was observed that miR-155 expression of a lentivirally transduced transgene of miR-155 was unaffected, and it was only when the 8-mer or both sites were mutated that the repression was completely abrogated, and miR-155 then had no effect on the reporter expression (Fig. 1). These results confirmed that SMAD2 is a direct target of miR-155 and that the 8-mer seeding region for miR-155 is the most relevant for the microRNA to exert its repressing action.
levels increased over 96 h by 3-fold when compared with the untreated control cells (Fig. 2A). In cells overexpressing miR-155, SMAD2 protein levels were repressed by 63% after 96 h (Fig. 2B, upper panel), whereas SMAD2 mRNA levels remained unchanged (Fig. 2B, upper panel) compared with control cells. We also performed a control experiment on the same cell lysates, determining the levels of SMAD3 and SMAD4 proteins after miR-155 induction. SMAD3 and SMAD4 levels were unaltered by the overexpression of miR-155 (supplemental Fig. S2 and S3), showing that the effect of miR-155 on SMAD2 is specific. These results show that an increase in miR-155 can significantly reduce the levels of SMAD2 protein in macrophages. Furthermore, because the mRNA levels of SMAD2 remain unchanged while the protein expression drops, the mechanism of action for miR-155 seems more likely to be based on repression of translation rather than degradation of its target mRNA. Taken together (Fig. 1), these findings demonstrate that SMAD2 is a direct target for miR-155 in macrophages.

Effect of miR-155 Overexpression on TGF-β1 Induction of SMAD2 Phosphorylation—After interaction of TGF-β with the heterodimeric TGF-β receptor complex consisting of the TGF-β receptor I together with the TGF-β receptor II, the activated TGFβRII induces phosphorylation of SMAD2. This allows the phosphorylated SMAD2 to shuttle into the nucleus where it associates with other transcription factors and binds to active promoters, positively or negatively affecting the transcriptional expression of a myriad of genes. After the demonstration that miR-155 down-regulates SMAD2 in our THP1–155 model, we predicted that this repression would reduce the levels of the functional, phosphorylated form of SMAD2 (phospho-SMAD2). The effect of miR-155 on SMAD2 protein levels (Fig. 2) should have no functional consequence if, after TGF-β1 stimulation, there is no change in the levels of phospho-SMAD2. We, therefore, went on to assess whether or not miR-155 repression of SMAD2 expression alters the levels of phospho-SMAD2 after TGF-β1 treatment. After treatment of THP1–155 cells with or without doxycycline for 96 h, cells were starved for a further 24 h and then treated with 10 ng/ml TGF-β1. Phospho-SMAD2 levels were quantified by Western immunoblotting at different time points spanning the first 3 h of induction. Over this period, up-regulation of miR-155 did indeed appear to affect the generation SMAD2 and its phosphorylated form phospho-SMAD2, the levels of which were reduced by 25, 60, and 80%, respectively, in the cells overexpressing miR-155. We also determined in parallel the levels of SMAD3 and phospho-SMAD3 to assess whether the effect of miR-155 on phospho-SMAD2 was specific and not due to an altered kinase activity of the TGF-β receptor complex. Phospho-SMAD3 and SMAD3 (23) levels were unaltered (Fig. 3, lower panel), showing that the upstream TGF-β signaling was not affected by miR-155 in our experiments. Our results showed that in our model miR-155 affects the levels of SMAD2 protein, and that as expected, this reduced the level of the phosphorylated, activated form of the transcription factor.

FIGURE 2. SMAD2 protein levels are down-regulated when THP1–155 cells overexpress miR-155. A, THP1–155 were treated or not with doxycycline (Dox) to de-repress or not the expression of miR-155 transgene, respectively. Cells were collected at different time points and subjected to Western blotting, and SMAD2 levels were determined. As a normalizing control, β-actin was also detected in the same blot. Values represent percentages of SMAD2 normalized against β-actin and compared with control. C, the same RNA was extracts was also used to perform a standard RT-qPCR to determine SMAD2 mRNA levels. Values were normalized against GAPDH.
miR-155 Regulates TGF-β Signaling by Targeting SMAD2

Effect of miR-155 on the TGF-β-dependent Transcriptional Activation—After phosphorylation and translocation into the nucleus, phospho-SMAD2 binds to specific promoters, enhancing their transcriptional activity. We have demonstrated that the levels of phospho-SMAD2 are reduced because of SMAD2 has, indeed, a very similar effect to that shown in Fig. 4, taking place at the promoter level. For this purpose, we chose to study the transcriptional activation of SERPINE1, a gene involved in fibrosis (23) and known to be activated by SMAD proteins after TGF-β stimulation (24). First, we used THP1–155 cells, treated for 96 h with or without doxycycline. This was followed by a 48-h treatment with TGF-β, after which cells were collected and SERPINE1 mRNA levels were determined by RT-qPCR. Fig. 4A shows that the TGF-β activation of SERPINE1 mRNA expression was reduced to a third when miR-155 was induced. To further confirm this result, THP1 cells were transfected with pre-microRNA oligonucleotides. Cells were transfected with pre-miR-155, pre-miR-221 (a control microRNA), or pre-miR control#1 (a scrambled microRNA). 48 h after the transfection, cells were treated or not with TGF-β. 48 h after the treatment, SERPINE1 mRNA expression was determined by RT-qPCR. Fig. 4B shows that pre-miR-155 transfection reduced the induction of SERPINE1 mediated by TGF-β, whereas the control pre-microRNAs did not affect it.

Furthermore, we wanted to determine whether the depletion of SMAD2 in THP-1 cells by RNAi could lead to the effects similarly to those that have been shown in Fig. 4, A and B. We then transfected THP-1 cells with siRNAs against SMAD2 (#1 and #2) or control and treated with TGF-β or not 48 h after transfection. Cells were collected before the TGF-β treatment to determine the efficiency of the SMAD2 knockdown. Supplemental Fig. S4 shows a clear reduction in the mRNA expression of SMAD2. 48 h after TGF-β treatment cells were collected, and SERPINE1 mRNA expression was determined as above. Fig. 4C shows the knockdown of SMAD2 has, indeed, a very similar effect to that shown in Figs. 4, A and B.

Finally, we wanted to confirm that the effect that we describe in Fig. 4A takes place at the promoter level. For this purpose, we used a synthetic reporter construct consisting of luciferase coupled to 12 SMAD binding sites that are present...
in the promoter of the SERPINE1 gene, CAGA12LUC. The transcriptional activity of this reporter is dependent on TGF-
β-activated SMAD proteins (both SMAD2 and SMAD3 heterodimerize with SMAD4). This construct was transfected into our monocytic cell model, THP1–155 to assess the effect of miR-155 on SMAD-dependent promoter activation. To de-repress or not the expression of the miR-155 transgene, THP1–155 cells were treated for 96 h with or without doxycycline. The cells were then transfected with the CAGA12LUC vector and pRLTK as a normalizing vector. After transfection, cells were starved for 12 h before the addition or not of TGFβ1 (10 ng/ml). Cell lysates were collected 24 h later and subjected to a dual luciferase activity assay. The results showed that TGF-β was able to enhance the transcriptional activity of the reporter ~5-fold when compared with the basal conditions in untreated cells (Fig. 4D). However, overexpression of miR-155 reduced TGF-β-dependent activation of CAGA12LUC transcription by ~40%, therefore, reflecting a partial abrogation SMAD activity. The remaining TGF-β-de-
dependent transcriptional activity (up to a 60% when compared with the control) is most probably accounted for by SMAD3 activity with a likely contribution by residual phospho-SMAD2 protein, as seen in Fig. 3. These experiments confirmed that the down-regulation of SMAD2 by miR-155 diminishes the ability of SMAD2 to mediate TGF-β-dependent activation of gene regulation (SERPINE1, Figs. 4, A, B, and C) and promoter activity (Fig. 4D). This conclusion has important consequences as TGF-β affects many other genes related to many different biological processes.

Effect of miR-155 on TGF-β-dependent Gene Expression—
We have proved that miR-155 down-regulates the levels of SMAD2 protein, which leads to less phospho-SMAD2 after TGF-β treatment and that this affects the ability of TGF-β to promote transcriptional activation. TGF-β is known to regulate a large number of genes related to different biological processes. Increase in TGF-β has been associated with the promotion of fibrosis and extracellular matrix deposition (18).

Also, TGF-β is known to modulate angiogenesis and to be an immunomodulatory cytokine. After showing the inhibitory effect of miR-155 on the TGF-β-dependent expression of SERPINE1(Fig. 4), we wondered whether the targeting of SMAD2 by miR-155 could allow this microRNA to have a wider effect and modify TGF-β/SMAD2-dependent gene expression and, thus, modulate all the biological responses to this cytokine. To assess the relevance of our findings on the biological activities of TGF-β, we studied the expression of an array of genes involved in these processes in our THP1–155 system; cells overexpressing miR-155 or not were stimulated or not with TGF-β. We chose genes that are TGF-β-depen-
dent and involved in fibrosis (TGF-β1, TGF-β2, TGF-β3), angiogenesis (VEGF, ICAM1, MMP9), and immunity (IL-1β, IL-4 receptor, macrophage migration inhibitory factor, and DC-SIGN). As the control, we also studied SMAD2 itself as it is unaffected by TGF-β stimulation. miR-155 expression was induced by treatment of the cells with doxycycline for 96 h, and then the cells were stimulated with TGF-β for 48 h. Cells were lysed, and RNA extracted and determined by RT-qPCR. We first confirmed that miR-155 levels were up-regulated (2.5-fold) after treatment with doxycycline and were not affected by TGF-β1 treatment (Fig. 5). When analyzing the results obtained by quantifying the expression of our set of genes, we encountered a range of different responses both to TGF-β and miR-155. The effects of TGF-β ranged from no effect (SMAD2, miR-155, TGF-β3, and macrophage migration inhibitory factor) to a 4–7-fold increase (TGFβ1, TGF-
β2, IL1β, ICAM1, MMP9, and VEGF). Between these extremes, we found a small effect on IL-4R and DC-SIGN (~1.7-fold increase). In all of the genes analyzed that were up-regulated by TGF-β, we observed that the TGF-β-induced stimulation was inhibited by miR-155. In some of these genes, we observed a complete abrogation of the TGF-β activation (TGF-β2, MMP9, ICAM1, IL-4R, and DC-SIGN), whereas others showed a partial inhibition ranging from a 60 to an 85% reduction of the TGF-β-induced activation (TGF-β1, VEGF, and IL-1β). Interestingly, for three genes (IL-1β, MMP9, and DC-SIGN) the basal expression levels were decreased after up-regulation of miR-155. Finally, neither TGF-β3 nor macrophage migration inhibitory factor appears to be up-regulated by TGF-β1 in these cells nor were they affected by up-regulation of miR-155. These results indicate a complex picture in which TGF-β-dependent genes are af-
fected by miR-155, which abrogates or partially inhibits the TGF-β-dependent stimulation and, in some cases, reduces the basal level of expression of some of these genes. Alto-
together, our data suggest an important role of miR-155 in sev-
eral biological activities of TGF-β.

DISCUSSION
TGF-β is a pleiotropic cytokine, with varied and important biological roles. Several human diseases have been described in which TGF-β dysregulation leads to pathological deposition of extracellular matrix, aberrant remodeling or cell proliferation, and uncontrolled angiogenesis (6, 25, 26). Mouse models have confirmed these roles, which are also crucial in development. As an example, SMAD3 KO mice manifest less excess of fibrotic processes (26) that are visible in the lung after treatment with TGF-β. Interestingly, asthma, a common respiratory disease of humans, also manifests fibrosis and re-
modeling processes, which correlate with a higher level of TGF-β in the lung (27). Thus, TGF-β is a key player in some of the most important biological roles that take place in the organism. We have presented data here confirming that miR-155 contributes to the regulation of the TGF-β signaling pathway through direct targeting of SMAD2, and this has a direct impact on the biological functions of this cytokine.

Our preliminary observation employing the bioinformatic tool Targetscan predicted that the 3′-UTR of SMAD2 con-
tained two seeding regions for miR-155. This observation sug-
gested the possibility of direct interaction between miR-155 and the TGF-β signaling pathway. To investigate this possibility, our first approach was to establish that miR-155 does directly target SMAD2 via the region of the 3′-UTR of SMAD2 that contains both targeting sequences for miR-155. We did this by fusing this region to the renilla luciferase gene and comparing the “wild type” sequence to constructs in which the seeding regions for miR-155 were mutated (Fig. 1). Our
data show that only the seeding region that presents an 8-mer complementarity (that is, a complementarity of 8 nucleotides between the mRNA and the 5' end of the microRNA) is functional, by blocking the expression of the SMAD2 3'-UTR-renilla luciferase construct (Fig. 1). Mutation of this sequence renders the 3'-UTR-renilla luciferase construct inaccessible to miR-155. On the other hand, mutation of the second binding site for miR-155, a 7-mer, is unable to protect the mRNA from being degraded by miR-155. These data show a prominent role for one of the binding regions of miR-155; similar results were obtained in a recent study on keratinocyte growth factor (KGF), where only one predicted site is relevant for repression by miR-155 (28). Both genes, kgf and smad2, contain in their 3'-UTRs two target sites for miR-155, an 8-mer and a 7-mer. Interestingly, both 8-mers have similar site type contribution value (−0.310, according to Targetscan), versus both 7-mers, which both have a worse contribution value (−0.099). In both studies only the 8-mer site really contributed to miR-155 repressing actions on the 3'-UTR of their respective target, keratinocyte growth factor, or SMAD2. This is in concordance with the prediction values made with the Targetscan software and might help future studies decide whether a specific miR target site is worth studying, taking into account the site type contribution value using the Targetscan algorithm (20).

Having established this molecular link, we investigated the role of miR-155 on TGF-β signaling in macrophages. Macrophages are typically involved in the defense against pathogens, participating in the initiation of inflammation, but they also have repair and pro-angiogenic roles in the resolution of the...
miR-155 Regulates TGF-β Signaling by Targeting SMAD2

inflammatory process (29). We employed the monocytic cell line THP1, often used as a macrophage-like surrogate. We had previously generated a stably transfected cell line, THP1–155 (17), employing a system of lentiviral vectors that allows doxycycline-inducible control of miR-155. In this cellular system we proved that an increase in the levels of miR-155 was able to reduce the protein levels of SMAD2 (Fig. 2) without affecting the levels of SMAD2 mRNA. This suggests that, in this system, miR-155 blocks translation of SMAD2 rather than promoting degradation of the mRNA. We have found this to be the case for other genes studied by our group, including PU.1 (17) and IL-13 receptor-α1 (currently under investigation). Together with SMAD3 and SMAD4, SMAD2 is a key component of the TGF-β signaling pathway, with important roles in the biological processes activated and controlled by this cytokine. As mentioned under “Results,” TGF-β promotes SMAD2 activation through phosphorylation. We determined whether this activation was affected by the overexpression of miR-155. Our data show that, whereas phosphorylation of SMAD2 occurred, it did not reach its maximum levels when miR-155 is up-regulated (Fig. 3). This is probably due to the limiting levels of SMAD2 protein available to TGF-β receptor I. However, it could also have been due to an upstream defect in the receptor system for TGF-β, but this was ruled out by the fact that SMAD3 phosphorylation was intact (Fig. 3, lower panel).

Phospho-SMAD2 exerts its role in the nucleus by binding to different gene promoters and activating or inhibiting their transcription. A reduction in phospho-SMAD2 levels suggests TGF-β/SMAD-dependent promoter activity and, thus, gene expression would be reduced. We proved this by showing that TGF-β-dependent gene expression of SERPINE1 was inhibited by miR-155 overexpression both in the THP1–155 system (Fig. 4A) as well as when transfecting a pre-miR-155 oligonucleotide (Fig. 4B). This inhibitory effect was mimicked by using an siRNA against SMAD2 (Fig. 4C), showing the implication of this transcription factor. We further analyzed the effect of miR-155 on the SMAD/TGF-β-dependent promoter activity by performing transfections in THP1–155 cells with a synthetic promoter containing 12 CAGA SMAD2/3/4-responsive elements in tandem. When miR-155 levels were up-regulated, TGF-β-induced activation of the promoter in this plasmid was reduced by ~50% (Fig. 4). This was less dramatic than the decrease in phospho-SMAD2 levels, 80% after 3 h TGF-β treatment (Fig. 3). The remaining TGF-β-dependent promoter activity in the synthetic promoter as well as in the gene expression of SERPINE1 is probably mediated by SMAD3 as well as the remaining phospho-SMAD2.

The evidence presented in Figs. 1–4 strongly suggests that miR-155 plays a role modulating the response of monocytes to TGF-β. To test the hypothesis that the presence of miR-155 could affect TGF-β-induced activation of a wide range of genes, we quantified the mRNA expression of several TGF-β-inducible genes. Our results show clearly that up-regulation of miR-155 in THP1–155 cells reduces the TGF-β-induced activation of a number of genes. However, these genes did not respond uniformly to the increase in miR-155 levels (Fig. 5). TGF-β-induced activation of gene expression ranging from 1.7- to 7-fold. Up-regulation of miR-155 had different effects on expression of different genes; whereas for some genes (MMP9, ICAM1, IL-4R, and DC-SIGN) there was complete abrogation of activation by TGF-β, for others (TGF-β1 and -2, VEGF, and IL-1(β) the fold increase in expression was reduced to 30 or 10%. These results do not perfectly match the decreases shown in Figs. 3 or 4D in which the reduction in phospho-SMAD2 and TGF-β/SMAD-dependent promoter activity was in the range of 20–50%, respectively; the effect on gene expression seems to be proportionately greater. Apart from the difficulty of accurately comparing quantification by different experimental techniques (Western blot, luciferase assay, and RT-qPCR), there are some other possible explanations for this discordance. While the luciferase assay mainly measures SMAD-mediated transcriptional activity, every individual gene might have a different promoter. SMAD2 could be essential for the TGF-β-dependent activation of certain promoters (MMP9, ICAM1, IL-4R, and DC-SIGN), whereas for other genes it might share its activity with SMAD3. Furthermore, as mentioned above, SMAD proteins act through cooperative complexes with other transcription factors. For example, SMAD2/3 form a complex with the hypoxia-inducible factor (HIF-1), which enhances the TGF-β-induced activation of the VEGF promoter (2, 30). Thus, it is conceivable that other transcription factors may be affected by miR-155, adding another layer of complexity to the control of genes activated by TGF-β. The fact that in the presence of increased miR-155, four of the genes studied showed decreased basal levels of expression (Fig. 5, IL-1β, MMP9, TGF-β2, and DC-SIGN) suggests that other pathways may also be affected by miR-155 and are, hence, unable to co-operate efficiently with TGF-β. We have already shown that the expression DC-SIGN is down-regulated by miR-155 through direct targeting and reduction of the transcription factor PU.1 (17). Also, it has been shown in dendritic cells that miR-155 affects IL-1(β) mRNA levels by direct targeting of TAB2, a cytoplasmic transcription factor intermediate in the NFκB pathway (31). Additionally, it has been shown recently that miR-155 targets SMAD5 in lymphocytes, affecting a non canonical TGF-β signaling pathway (32). SMAD5 is thought to mediate signaling of bone morphogenetic protein, but it can also affect TGF-β signaling and, thus, may be a candidate to explain the variability shown in the different genes studied in this work.

Our findings show a complex picture in which miR-155 affects a network of genes involved in different processes essential to the biological functions of macrophages. miR-155 does not seem to affect all these processes in the same way, probably fine-tuning their exact roles, depending on the biological need. Shutting down some genes while just decreasing others could be a very precise way of controlling essential processes such as fibrosis, angiogenesis, and immunity.

In conclusion, miR-155 could have a broad role in controlling TGF-β signaling pathways (among other functions), which makes this microRNA of particular interest when studying any of the diseases in which the above mentioned essential processes are involved. Understanding the subtleties behind these regulatory mechanisms might bring much needed insights into a wide range of pathological processes.
miR-155 Regulates TGF-β Signaling by Targeting SMAD2

There is a growing realization that many diseases such as rheumatoid disease (33), asthma (27), and inflammatory bowel disease (34) appear to involve critical impairment in the homeostatic controls exerted by TGF-β. The findings presented here indicate a fundamental role for microRNAs as the agents responsible for the ultimately pathological loss of TGF-β mediated control.

Acknowledgment—We thank Professor Peter Friedmann for critical review of the manuscript and for help in the discussion of the results.

REFERENCES

1. Massagué, J. (2000) Nat. Rev. Mol. Cell Biol. 1, 169–178
2. Sánchez-Elsner, T., Botella, L. M., Velasco, B., Corbí, A., Attisano, L., and Bernabéu, C. (2001) J. Biol. Chem. 276, 38527–38535
3. Khalil, N., Parekh, T. V., O’Connor, R., Antman, N., Kepron, W., Ye, B. and Gold, L. I. (2001) Annu. Rev. Biochem. 70, 334–340
4. Padua, D., and Massagué, J. (2009) Cell Res. 19, 89–102
5. Martin, M. M., Lee, E. J., Buckenberger, J. A., Schmittgen, T. D., and Durand, A. (1999) J. Immunol. 162, 4567–4575
6. Lai, E. C. (2002) Nat. Genet. 30, 363–364
7. Brennecke, J., Stark, A., Russell, R. B., and Cohen, S. M. (2005) PLoS Biol. 3, e85
8. Bushati, N., and Cohen, S. M. (2007) Annu. Rev. Cell Dev. Biol. 23, 175–205
9. Martin, M. M., Lee, E. J., Buckenberger, J. A., Schmittgen, T. D., and Elton, T. S. (2006) J. Biol. Chem. 281, 18277–18284
10. Kong, W., Yang, H., He, L., Zhao, J., J., Coppola, D., Dalton, W. S., and Cheng, J. Q. (2008) Mol. Cell Biol. 28, 6773–6784
11. Tili, E., Croce, C. M., and Michaille, J. J. (2009) Int. Rev. Immunol. 28, 264–284
12. O’Connell, R. M., Taganov, K. D., Boldin, M. P., Cheng, G., and Baltimore, D. (2007) Proc. Natl. Acad. Sci. U.S.A. 104, 1604–1609
13. Martinez-Nunez, R. T., Louafi, F., Friedmann, P. S., and Sanchez-Elsner, T. (2009) J. Biol. Chem. 284, 16334–16342
14. Kong, W., Yang, H., He, L., Zhao, J. J., Coppola, D., Dalton, W. S., and Cheng, J. Q. (2008) Mol. Cell Biol. 28, 6773–6784
15. Tili, E., Croce, C. M., and Michaille, J. J. (2009) Int. Rev. Immunol. 28, 264–284
16. O’Connell, R. M., Taganov, K. D., Boldin, M. P., Cheng, G., and Baltimore, D. (2007) Proc. Natl. Acad. Sci. U.S.A. 104, 1604–1609
17. Martinez-Nunez, R. T., Louafi, F., Friedmann, P. S., and Sanchez-Elsner, T. (2009) J. Biol. Chem. 284, 16334–16342
18. Stanczyk, J., Pedrioli, D. M., Brentano, F., Sanchez-Pernaute, O., Kolling, C., Gay, R. E., Detmar, M., Gay, S., and Kyburz, D. (2008) Arthritis Rheum. 58, 1001–1009
19. Jiang, S., Zhang, H. W., Lu, M. H., He, X. H., Li, Y., Gu, H., Liu, M. F., and Wang, E. D. (2010) Cancer Res. 70, 3119–3127
20. Lewis, B. P., Shih, I. H., Jones-Rhoades, M. W., Bartel, D. P., and Burge, C. B. (2003) Cell 115, 787–798
21. Dennler, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S., and Gauthier, J. M. (1998) EMBO J. 17, 3091–3100
22. Li, M. O., Wan, Y. Y., Sanjabi, S., Robertson, A. K., and Flavell, R. A. (2006) Annu. Rev. Immunol. 24, 99–146
23. Senoo, T., Hattori, N., Tomimata, T., Furuoka, M., Ishikawa, N., Fujitaka, K., Haruta, Y., Murai, H., Yokoyama, A., and Kohno, N. (2010) Thorax 65, 334–340
24. Min, A. K., Kim, M. K., Seo, H. Y., Kim, H. S., Jang, B. K., Hwang, J. S., Choi, H. S., Lee, K. U., Park, K. G., and Lee, I. K. (2010) Biochem. Biophys. Res. Commun. 393, 536–541
25. ten Dijke, P., and Arthur, H. M. (2007) Nat. Rev. Mol. Cell Biol. 8, 857–869
26. Bonniaud, P., Kolb, M., Galt, T., Robertson, J., Robbins, C., Stampfl, M., Lavery, C., Margetts, P. J., Roberts, A. B., and Gauldie, J. (2004) J. Immunol. 173, 2099–2108
27. Bosse, Y., and Rola-Pleszczynski, M. (2007) Respir. Res. 8, 66
28. Pottier, N., Maurin, T., Chevalier, B., Puissegur, M. P., Lebrigand, K., Robbe-Sermesant, K., Bertero, T., Lino Cardenas, C. L., Courcot, E., Rios, G., Fourre, S., Lo-Guidice, J. M., Marcit, B., Cardinaud, B., Barbry, P., and Mari, B. (2009) PLoS One 4, e6718
29. Parmar, A., Eubank, T. D., and Dosef, A. I. (2010) J. Innate Immun. 2, 204–215
30. Shih, S. C., and Claffey, K. P. (2001) Growth Factors 19, 19–34
31. Ceppi, M., Pereira, P. M., Dunand-Sauvigny, I., Barras, E., Reith, W., Santos, M. A., and Pierre, P. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 2735–2740
32. Rai, D., Kim, S. W., McKeller, M. R., Dahia, P. L., and Aguiar, R. C. (2010) Proc. Natl. Acad. Sci. U.S.A. 107, 3111–3116
33. Pohlers, D., Brennechi, I., Löffler, I., Müller, C. K., Leipner, C., Schulz-Mosgau, S., Stallmach, A., Kinne, R. W., and Wolf, G. (2009) Biochim. Biophys. Acta 1792, 746–756
34. Monteleone, G., Kumberova, A., Croft, N. M., McKenzie, C., Steer, H. W., and MacDonald, T. T. (2007) J. Clin. Invest. 108, 601–609
35. Ralser, M., Querfurth, R., Wernat, H. J., Lehrach, H., Yaspo, M. L., and Krobitsch, S. (2006) Biochem. Biophys. Res. Commun. 347, 747–751