Published in final edited form as:

Exp Cell Res. 2007 October 15; 313(17): 3718–3728.

Promoter-independent regulation of vimentin expression in mammary epithelial cells by val¹²*ras* and TGF β

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Abstract

The 1029 series of mammary epithelial cell lines (D6, GP+E, r3 and r3T) are progressively more transformed: the latter two by val¹² *ras*. These cell lines respond to TGF β by undergoing early events of epithelial-mesenchymal transition (EMT), including morphological changes and redistribution of E-cadherin. Tumors formed by r3T cells in the choroid of the eye express vimentin, a late marker of EMT, possibly in response to TGF β . In vitro, vimentin expression is induced in all the cell lines by TGF β treatment, while cytokeratin expression is only slightly affected. Surprisingly, *ras* transformation results in a 10-fold suppression of vimentin expression. Neither suppression of vimentin by *ras* transformation nor induction by TGF β are mediated by the vimentin promoter in r3T cells. In transient transfection assays, several human vimentin promoter constructs are more active in the low-expressing r3T cell line than in the vimentin-expressing mesenchymal cell line NIH3T3. In the r3T cells, there is no effect of TGF β treatment for 9 days on the activity of either promoter. Azacytidine treatment does not affect vimentin expression in either NIH3T3 or r3T, suggesting that promoter methylation is not the mechanism of suppression by *ras*. Finally, the half life of the vimentin mRNA is similar in both the r3T cells and NIH3T3 cells. We conclude that the suppression of vimentin expression by *ras*, and the relief of this suppression by TGF β , occurs in a promoter-independent fashion, possibly through sequences in the first or second intron.

Keywords

EMT; TGF β ; vimentin; suppression; metastasis

Introduction

The epithelial mesenchymal transition (EMT), in which epithelial cells take on various characteristics of the mesenchymal lineage, occurs normally during embryonic development, organogenesis and wound healing [1]. An excellent example of EMT during development is palatal fusion, in which fusion of the mesenchymal tissues occurs after conversion of the separating surface epithelium to mesenchyme by EMT [2]. This same transition frequently accompanies the development of metastatic tumors, and correlates with increased tumor

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aggressiveness [3,4]. EMT is characterized by the rearrangement of several membrane associated proteins, including E-cadherin and ZO-1, as well as changes in cytoskeletal organization. Upregulation of vimentin expression is a particularly well-known marker of the EMT, and expression of vimentin in human epithelial cancers is frequently observed [5,6] and has been suggested as a prognostic marker [7,8].

In culture, cells undergo the EMT in response to a variety of stimuli, of which TGF β stimulation is well characterized [9–11]. In normal epithelial cells, TGF β blocks cell proliferation, in addition to causing EMT, while transformed cells are generally resistant to the inhibition of proliferation. Many transformed epithelial cells in culture have already partially or completely undergone the EMT, but there are also metastatic cell lines that retain the epithelial phenotype. Despite its role in blocking the development of epithelial cancers [12], TGF β is known to stimulate growth of metastatic cancers, and the increased migration and invasion caused by TGF β likely contribute to the metastatic phenotype (reviewed in [13–15]).

Induction of EMT by TGF β has been linked to *ras* expression [10,11], and the timing of EMT-linked events has been characterized in *ras* transformed cells. The morphological changes and disruption of cell–cell interactions occurs rapidly (within 24 hours): this process is termed scattering. Later events including loss of polarity and increased vimentin expression require several days to complete [16].

The regulation of vimentin expression in mesenchymal cells has been well studied, and transcription factor binding sites responsible for serum stimulation and silencing have been described [17–21]. In epithelial cells, vimentin expression is higher in migratory cells, and may contribute to the migratory and invasive phenotype of metastatic cells [21]. However, the mechanism of vimentin transcriptional regulation during the EMT is poorly understood. We have developed a series of transformed mouse mammary epithelial cell lines with progressive degrees of transformation [22]. All these cells maintain an epithelial phenotype in culture, and undergo EMT in response to TGF β [23]. Here we explore the regulation of vimentin expression in these cells in response to *ras*-transformation and to TGF β , and demonstrate that the regulation is complex, and possibly not controlled by the well-characterized proximal promoter.

Materials and Methods

Cell lines and culture

Mammary epithelial cells D6, GP+E, r1,r3 and r5 and r3T [22] were cultured in α MEM with 8% FBS. Selection in 3 μ g/ml puromycin (all cells except D6) and 800 μ g/ml G418 (r3 and r3T) was maintained continuously in culture. Cells were treated with TGF β (Peprotech, Rocky Hill) at 100 pM in complete medium for the indicated times. NIH3T3 cells were grown in DMEM with 10% calf serum. All cells were passed every three or four days. DRB (5,6-Dichloro-1- β -D-ribofuranosylbenzimidazole Sigma, St Louis) was dissolved in DMSO at 1 mg/ml, and stored at -20°C . SD093 was provided by Scios, Inc (Fremont, CA) and was dissolved in DMSO at 10 mM [24]. 5-Azacytidine was dissolved in sterile PBS at 10mM and used at a final concentration of 10 μ M, the maximal concentration that showed no cytotoxicity in the NIH3T3 cells. Cells were transfected with lipofectamine 2000 at confluence (r3T) or subconfluence (NIH3T3) at a DNA:lipid concentration of 1:0.5. Cells were cotransfected with equal amounts (800 ng/24 well) of luciferase construct and pRLTK (Promega, Madison) expressing Renilla luciferase. Cells were harvested 24 hours after transfection, and luciferase activity was measured using the Dual-Glo luciferase assay kit (Promega). CAT activity was measured in a scintillation counter using the CAT Activity kit (Promega).

Northern Blotting

RNA was prepared using Trizol. Five μ g RNA were separated on 1% agarose gels in MOPS buffer with formaldehyde as described [25] and transferred to Genescreen Plus. DNA fragments were labeled with 32 P by random priming, and hybridized by standard techniques. Signals were detected by autoradiography or by Typhoon imaging. The vimentin probe was human cDNA [26]. The cytokeratin 18 probe was generated by PCR amplification from D6 cells.

Western blotting

was performed as described [27]. Antibodies used were mouse anti-cytokeratin 18 (Chemicon), mouse anti-vimentin (Abcam) and mouse anti- β -actin (Sigma). Antibody binding was detected with HRP labeled goat anti-mouse IgG (Bio-Rad). The Smad-2-P specific antibody was previously described [28]; total Smad-2 was detected with a rabbit polyclonal (Zymed, South San Francisco, USA). Immunohistochemistry for vimentin and cytokeratin was performed using the same cytokeratin antibody as for western blotting, and a goat anti-mouse vimentin antibody (Santa Cruz Biotechnology) at 1:500 and 1:1000, respectively. Following antigen retrieval, sections were blocked with 1% BSA, 1:20 serum for one hour and then incubated with primary antibody for one hour. Biotin labeled secondary antibodies were applied, followed by the ABC reagent (Vector). Antibody reactivity was detected with diaminobenzidine (Sigma Fast DAB). Slides were lightly counterstained with hematoxylin. Immunofluorescence for E-cadherin was performed using a monoclonal anti-mouse antibody (BD, Bedford MA), which was detected with a CY3 conjugated secondary antibody.

Vimentin promoter constructs

Human vimentin promoter constructs driving chloramphenicol acetyl transferase (CAT) expression were previously described [17]. The -411 promoter fragment was excised from a CAT construct and cloned into pGL3 basic (Promega) luciferase construct. This vector was subsequently digested at the XhoI site at -150 of the vimentin promoter, and at an upstream SacI site in the vector, and a 4 kb fragment of the human vimentin promoter was inserted. This 4 kb fragment was obtained by SacI-XhoI digestion of human BAC RP11 124N14 (CHORI, Oakland, CA) containing a 94 kb fragment of human DNA including the vimentin gene. Appropriate clones were identified by restriction analysis.

QPCR and semiquantitative PCR

Primers were as follows: mouse vimentin F : cggaagtgaatccttgca, mouse vimentin R: cacatgatctggacatgctgt, GAPDH F: gcaaagtgagattgtgcat, GAPDH R: ccttgactgtgccgtgaattt, DNMT2 F: gtccagaaggtccatgtct, DNMT2 R: ggtggcaatcaggaagaga. qPCR (quantitative, real time PCR) was performed on an ABI7900 HT system or a BioRad ICycler, using a master mix (BioRad) containing Sybr Green and Rox or fluorescein, respectively. cDNA was generated from total RNA in a 40 μ l reaction, and 1–4 μ l of cDNA was used for qPCR, depending on the abundance of the target. For vimentin and GAPDH, linearized plasmid DNA was used to generate a standard curve, and numbers of molecules of RNA per sample was calculated. For DNMT2, the relative levels of mRNA were calculated based on dilutions of a single cDNA to generate the standard curve. The efficiency of the PCR reaction was 98.3% for vimentin, 80% for GAPDH, and 70% for DNMT2. In each case, experimental values were normalized to β -2 microglobulin (Figure 3B) or glyceraldehyde phosphate dehydrogenase (GAPDH) levels in the same samples. Controls without reverse transcriptase were assessed for all samples.

Statistical Analysis

Experiments were performed in duplicate and/or multiple times as indicated, and data are presented as either representative results or mean $\pm$ SD. Where representative results are

shown, the results were confirmed in several experiments. Graph Pad Prism software was used to calculate standard deviations and p values.

Results

The 1029 series of mouse mammary cell lines has been described in detail [22]. Briefly, the D6 cells were isolated from a mammary tumor induced by DMBA and were cloned by limiting dilution. The GP+E cells were derived from the parental D6 cells by transformation with polyoma middle T. GP+E cells were further transformed with val^{L2} *ras*, giving rise to clones r1, r3, and r5. Finally the r3T cells were derived from a mammary tumor resulting from injection of r3 cells. The D6 and GP+E cells do not form tumors in syngeneic mice, while the remaining cell lines form metastatic tumors when injected into the mammary fat pad, and bone and choroidal metastases when injected into the arterial circulation [29,30].

All the cells of the D6 series have a strong epithelial phenotype in culture, displaying a cobblestone morphology, and growing as “islands” of closely apposed cells (Figure 1A, left panels). The D6 cells form domes in culture at high densities [22], further confirming their epithelial morphology. Upon exposure to TGF β , all the cell lines undergo the scattering phenomenon, losing their close connections and becoming spindle shaped (Figure 1A, right panels, and data not shown). All four cell lines also phosphorylate Smad-2 in response to TGF β (Figure 1B) Other phenotypic changes that occur in these cells as a result of TGF β treatment include rearrangement of the actin cytoskeleton to form prominent stress fibers (data not shown), and relocalization and suppression of E-cadherin. In untreated cells, E-cadherin is localized almost exclusively to the membrane of each cell line (arrows in Figure 1C, left panels); after TGF β treatment for 48 hours, E-cadherin is more diffusely distributed in the perimembranous region (white arrowheads). All these phenotypic changes are associated with the epithelial-mesenchymal transition, and all occur within 48 hours. Importantly, there is no effect of *ras* transformation on these TGF β -induced changes. These results confirm that TGF β signaling through the smad-2 pathway is intact in the r3T cells, and that typical changes in cell morphology occur in response to treatment with this cytokine.

Upon injection into the arterial circulation of syngeneic mice, the r3T cells form metastatic lesions primarily in the bone and the choroid of the eye [22,30]. Since vimentin expression is frequently observed in advanced human cancers, we asked whether metastatic lesions derived from r3T cells also expressed vimentin. Immunohistochemical analysis of choroidal metastases shows strong staining for vimentin (Figure 2), as well as for cytokeratin 18, reflecting the epithelial origin of these tumors. Since TGF β is associated with the development of metastatic lesions, we asked whether this growth factor is involved in the expression of vimentin in the choroidal metastases. It is known that TGF β is highly expressed in the nearby retinal pigment epithelial cells [31]; accordingly, the metastases arising in the choroid of the eye stain with an antibody that recognizes the phosphorylated form of Smad-2 (Figure 2). Phosphorylation of Smad-2 by the TGF β receptor is an indication of TGF β signaling in cells and tissues [13], so these data are consistent with the idea that vimentin expression in these metastases is induced by TGF β signaling.

These observations prompted us to ask whether TGF β induces vimentin expression in the 1029 series of cells in vitro. The in vitro phenotypic responses to TGF β documented in Figure 1 occur within 48 hrs. Induction of vimentin expression on the other hand, requires longer times, with expression increasing up to 144 hours after TGF β treatment (Figure 3A,B) in the r3T cells. Induction of vimentin expression by TGF β ranged from 2.3–3.1 fold in the different cell lines. Expression of vimentin protein was regulated in parallel with the mRNA: the protein was below the level of detection in all four cell lines under basal conditions, but was upregulated by TGF β treatment for 6 days (Figure 3C). Basal expression of vimentin (in the absence of

TGF β) was assessed by the more sensitive qPCR technique, demonstrating a more than 10-fold lower expression level in the *ras* transformed r3 and r3T cells than in the D6 and GP+E cells (Figure 3D). That this effect is not due to clonal variability affecting gene copy number or position effects is shown by the similar low-level expression of vimentin in three independent *ras* transformed clones (r1, r3 and r5; Figure 3D). These observations suggest that oncogenic *ras* suppresses vimentin expression in these epithelial cells.

Vimentin expression is minimal until 144 hr (6 days) incubation with TGF β in the r3 and r3T cells, as assessed at the mRNA level by northern blotting. Cytokeratin 18 expression, on the other hand, is only slightly affected by TGF β treatment (Figure 4A and B). This is consistent with the continued expression of cytokeratin in metastatic lesions thought to have undergone EMT in response to TGF β (Figure 2), and suggests that vimentin and cytokeratin 18 are not reciprocally regulated in these cells. Cytokeratin expression is not detectable in NIH3T3 fibroblast-like cells (Figure 4 A and B).

In order to begin to understand the mechanism of the regulation of vimentin expression in these epithelial cells, we first focused on the mechanism of suppression of vimentin expression by *ras* in the absence of TGF β . Both r3 and r3T cells were used for these analyses, since they have similar levels of vimentin mRNA expression: we compared these cells to NIH3T3, which express 100-fold higher levels of vimentin in the absence of TGF β (Figure 3D). The human vimentin promoter has been characterized in a variety of fibroblasts, and the well studied 1.5 kb promoter contains both enhancer and silencer elements [17,19,20]. We initially examined three promoter constructs containing 1500, 411 and 268 nucleotides of promoter sequence fused to a CAT reported gene. A strong enhancer comprising two AP-1 binding sites maps to the region between -1500 and -411 [17,18], several silencer elements are found between -411 and -268 [20,32], and weaker transcriptional stimulating sequences are located downstream of nucleotide -268. Surprisingly, when these constructs were transfected into r3 and NIH3T3 cells, CAT activity was about 3-fold higher in the r3 cells as compared to NIH3T3 cells, although the relative pattern of expression reported in the fibroblast cells was maintained (Figure 5A). This result suggests that the suppression of vimentin expression seen in the r3 cells is not due to transcription factor binding in this proximal promoter region. To test whether sequences upstream of the 1.5 kb promoter were required for suppression of vimentin transcription in the r3 cells, a fragment of the vimentin promoter extending about 4 kb (-4000) upstream of the transcription start site was cloned into a luciferase reporter vector (Figure 5B). Again, this promoter directed higher luciferase activity in the low-expressing r3 and r3T cells than in the high expressing NIH3T3 cells. In r3, r3T and the NIH3T3 cells, the normalized activity of the 4 kb promoter construct was about half that of the 411 nucleotide construct (Figure 5C). The results shown are representative of more than three independent experiments, and clearly demonstrate that the suppression of vimentin expression in the epithelial r3/r3T cells occurs at sequences other than the 4 kb promoter. Finally, we tested the effect of TGF β on activity of the -4000 and -411 nucleotide promoters in the r3T cells. r3T cells were cultured with or without TGF β for 9 days at which time endogenous vimentin expression is upregulated about 2.5 fold (Figure 3B and data not shown), and then transfected with the different vimentin promoter constructs (Figure 5D). There is no effect of TGF β on the activity of these transfected promoters, suggesting that induction of vimentin expression by TGF β in the r3T cells is mediated by the same promoter-independent mechanism used by *ras* to suppress vimentin expression.

Gene silencing can occur through methylation of promoter sequences[33,34], and methylation frequently does not occur on episomal plasmids present in transiently transfected cells. *ras* transformation has been shown to induce silencing of several genes such as RECK and clusterin [35–37]. Thus, methylation of the vimentin promoter resulting from *ras* transformation could explain the inability of the transfection experiments to reproduce the silencing effect of *ras*.

To determine whether the vimentin gene is silenced in the r3T cells by promoter methylation, these cells were cultured in the presence of the methylation inhibitor 5-azacytidine for up to four days. Analysis of vimentin expression by qPCR in these cells indicated no increase in expression in either r3T or NIH3T3 cells after azacytidine treatment (Figure 6A). Thus, we conclude that methylation of the vimentin gene is not the mechanism of silencing in the r3T cells. In addition, we used qPCR to measure the expression of the DNMT2 gene, which resides about 20 kB upstream of vimentin on chromosome 2. The expression of this gene was similar in r3T and NIH3T3 cells (data not shown), suggesting that large scale chromosomal silencing events are not responsible for the difference in vimentin expression between these cell lines.

Finally, to confirm that the suppression of vimentin expression in the r3T cells occurs at the level of transcription, we compared the half-life of vimentin mRNA in r3T and NIH3T3 cells. These cells were treated with DRB to block transcription, and vimentin mRNA levels measured after 3, 6, and 9 hours (Figure 6). The half life of vimentin mRNA is difficult to calculate in this experiment, since the half life of the normalizing gene, GAPDH, is similar to that of vimentin, making the slopes of the curves very shallow. Without normalization, the half life of vimentin mRNA is calculated as around 14 hours in NIH3T3 and approx 20 hours in r3T cells. The slopes of the half-life curves are not significantly different between the two cell lines with or without normalization ($p = .152$ and $.158$, respectively). Taken together, these results confirm that suppression of vimentin expression in the r3T cells does not occur at the level of message stability.

Discussion

Here we have shown that TGF β induces the epithelial-mesenchymal transition in a series of transformed mammary epithelial cells. Morphological changes in cell shape are accompanied by down-regulation and rearrangement of E-cadherin away from the membrane. Vimentin expression is also induced by TGF β in all these cell lines, but is a very late event, particularly in the *ras* transformed cells. Consistent with observations in the EpRas cells [16], the initial scattering or morphological change induced by TGF β in the 1029 series of cell lines precedes induction of vimentin by 1–4 days, depending on the particular cell line. The long time required for induction of vimentin expression in the r3T cell line in particular was unexpected, and contrasts with the rapid increase of expression seen in other epithelial cell types [21]. The reason for the requirement of extended exposure to TGF β for induction of vimentin expression in the r3 and r3T cells is unclear, but this observation suggests that vimentin is not a direct target of TGF β through Smad signaling. It may be that regulation of several genes in sequence may be required for vimentin upregulation: for instance upregulation of PDGF by TGF β in mammary epithelial cells requires 36 hours [38], and this upregulation of PDGF is thought to contribute to the induction of EMT [39]. Alternatively, TGF β upregulates HMG2A, which regulates chromatin structure: expression of HMG2A in turn induces Snail, Slug and Twist [40]. A similar transcription factor cascade may lead to vimentin induction by TGF β .

Janda and coworkers have suggested that *ras* expression is required for maintenance of the EMT, including late events such as loss of polarization and vimentin expression, and show that autocrine TGF β signaling is induced by *ras* in the EpH4 cell series [41]. This may not be the case in the D6 series of cells, as a clear induction of vimentin mRNA occurs in response to TGF β in both the D6 and GP+E cells, which as far as we know, are not *ras*-transformed. These cells do arise from a mammary tumor, however, and it is possible that the *ras* signaling pathway is upregulated in these cells. The dramatic effect of transfection of these cells with activated *ras*, however, including the ability to grow in soft agar and to form metastatic tumors [22], argues that the D6 and GP+E cells are not already *ras* transformed. On the other hand, there is a slight increase in the level of endogenous Smad-2 phosphorylation in the *ras*-transformed cell lines, which may indicate increased autocrine TGF β signaling in these cells.

The least transformed epithelial cell lines we examined, D6 and GP+E have surprisingly high basal levels of vimentin mRNA, only 4–5 fold lower than that seen in mesenchymal cells such as NIH3T3. Vimentin is expressed in epithelial cells that have already undergone the EMT, such as MDA-MB-231 and HeLa [42,43]; the 4T1 mouse epithelial cells also express vimentin [44]. The D6 and GP+E cells, in contrast to these other cell lines, do not show phenotypic evidence of EMT as evidenced by their epithelial morphology and E-cadherin distribution. Thus, it is unclear whether the basal vimentin expression seen in these cells reflects progression of these cells towards the mesenchymal phenotype. Interestingly, the non-transformed MDCK epithelial cells [45] also express vimentin, suggesting that this upregulation might reflect part of the adaptation of epithelial cells to culture. Indeed, Pieper and coworkers demonstrated that induction of vimentin expression is common in cells adapted to culture [46], a conclusion supported by other workers [47]. Vimentin protein, on the other hand, is not detected in unstimulated D6 and GP+E cells, suggesting that there may be some degree of translational control in these cells, although the very different sensitivities of western blot and qPCR make it difficult to compare these results directly.

The clear suppression of vimentin expression by *ras* transformation is striking. We observed similar levels of vimentin expression in three independent *ras*-transformed clones derived from the GP+E cells, so the reduced vimentin expression is unlikely to be due to clonal variability. Suppression of gene expression by activated *ras* has been described in NIH3T3 cells, where *ras* suppresses EGR-1 expression [48], and in human mammary epithelial cells where *ras* suppresses thrombospondin-1 expression [49]. In addition, transcriptional profiling of *ras* transformed cells reveals suppression of some genes, [50] so this observation is not unprecedented. However, this result is not consistent with the positive role of *ras* in supporting EMT reported by others [10]. It is possible that the presence of polyoma middle T in the r3 and r3T cells, in stimulating the PI3 kinase pathway, affects the normal signaling pathways in the *ras* transformed cells, leading to suppression of vimentin expression.

The independence of vimentin expression from the promoter sequence in transient transfection assays is also a striking and unexpected result. While vimentin mRNA levels determined by qPCR are 100-fold higher in NIH3T3 cells than in r3T cells, the vimentin promoter introduced by transient transfection is actually less active in the NIH3T3 cells than in the r3T cells. There are numerous studies describing transcription factor binding sites in the vimentin promoter, including those bound by the AP-1 complex [17] and TCF/LEF binding sites [21]. There are also a number of silencing elements identified in the promoter [17,20,51]. All these sites map to within the 1.5 kb promoter [18], and have been identified by transient transfection assays. In contrast, we have shown that this same 1.5 kb promoter is not subject to the same suppression in *ras* transformed cells as the endogenous gene. To test whether sequences further upstream may be responsible for the *ras*-induced suppression, we tested a larger 4 kb promoter fragment. This fragment directed about 40–60% less transcription than the 1.5 kb fragment, but still the activity of this promoter was similar in the NIH3T3 cells and in the r3/r3T cells. Thus, we conclude that suppression of vimentin transcription by *ras* does not occur through sequences 5' to the gene. While vimentin expression is 100 fold lower in r3T cells than in NIH3T3 cells, expression is still quantifiable in the r3T cells by quantitative PCR. We took advantage of this technique to demonstrate that the half life of vimentin mRNA is similar in both cell lines, and that promoter methylation is not involved in the suppression of vimentin expression by *ras*.

In these experiments, we used the human vimentin promoter to test expression in mouse cells. That the vimentin promoter sequence is functionally conserved is shown by the identification of numerous transcription sites on the human promoter in mouse cells [17,18,52,53] and in the chicken promoter in mouse and human cells [19,32,51]. Transgenic mouse studies also suggested that regulation of the human promoter in mouse cells and tissues parallels the endogenous gene [47]. However, the possibility remains that the human vimentin promoter is

not regulated correctly in the mouse epithelial cell lines we have examined. Experiments are underway to confirm these results using the mouse vimentin promoter.

The transcriptional repressors Snail, Slug and E47 are instrumental in the development of EMT. In MDCK cells, these transcription factors alone can induce EMT [54], and Snail expression is induced by TGF β [45,55] as well as during HGF-mediated EMT [56]. While these molecules are known as transcriptional repressors, array analysis of stably transfected MDCK cells shows that many genes are induced by Snail, Slug and E47 [57]. Is it possible that these factors are responsible for the suppression of vimentin expression by *ras*, or its induction by TGF β ? Vimentin was not among the genes identified as regulated by these factors in MDCK cells [57]. However, it is possible that one of these factors acts through sequences in the vimentin gene itself to regulate transcription. Many of these transcription factors bind to E-box sequences, however, and no E-box binding sites were identified by in silico analysis of the vimentin promoter and first intron (Rittling, S.R., unpublished data). On the other hand, vimentin expression in MCF10A cells is induced by the transcriptional repressor Smad-interacting protein (SIP-1) [21], which also down-regulates E-cadherin and is induced by TGF β [58]. Again, SIP-1 transactivates the human vimentin promoter, arguing against its involvement in the promoter-independent regulation of vimentin we have observed.

We have shown that the suppression of vimentin expression in the r3T cells does not occur through the most common mechanisms of gene regulation – transcriptional regulation through the promoter, regulation of mRNA stability, or promoter methylation. Large-scale chromosomal silencing events are also unlikely, since the nearby gene DNMT2 is expressed at similar levels in the r3T and NIH3T3 cells. Thus, we suggest that the suppression of vimentin by *ras*, and induction by TGF β , occurs through sequences in the introns or in the 3' end of the vimentin gene. Indeed, we have identified three regions in the first and second introns of vimentin that share high levels of homology (90–96%) between the mouse and the human vimentin genes. Interestingly, Sommers et al [26] in examining vimentin expression in expressing MDA-MB-231 cells and the non-expressing MCF7 cells identified several DNase hypersensitive sites in the first and second introns of the human vimentin gene. These sequences may correspond to the conserved sequences we have identified. Upregulation of N-cadherin by Twist, also a manifestation of EMT, is mediated through an E-box in the first intron [59], and there are regulatory regions in the E-cadherin first intron revealed through transgenic analysis [60]. Thus, intronic gene regulation may be a common theme in EMT. Work is currently underway to test the contribution of the intronic sequences to vimentin suppression in *ras*-transformed cells.

In conclusion, we demonstrate here for the first time that *ras* transformation leads to silencing of vimentin expression in mammary epithelial cells that maintain an epithelial phenotype in vitro. Neither this silencing, nor the subsequent up-regulation of vimentin expression by TGF β is mediated by the vimentin promoter, at least in transient transfection experiments, suggesting that sequences within the body of the vimentin gene, likely in the first or second intron, confer this regulation on the vimentin gene. This is in contrast to recent studies showing that TGF β stimulates the vimentin promoter in C2C12 myoblasts through the AP1 binding sites [53]. Future work to identify the sequences and associated transcriptional regulators that mediate the response to *ras* and TGF β will be important to fully understand the epithelial-mesenchymal transition and its contribution to metastasis.

Acknowledgements

We thank Vy Nguyen for valuable help with transfections, Tajneen Natasha for help with lab techniques, and the members of the Rittling lab for fruitful discussions. Supported by NIH #DK067685.

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Figure 1.

EMT occurs in all cell lines in response to TGFβ. **A:** Morphological changes in epithelial cells after 48 hours of TGFβ treatment. Cells were treated with 100 pM TGFβ (right panels) or vehicle only (left panels) for 48 hours and were then photographed under phase contrast illumination. Original magnification 10× **B:** Phosphorylation of Smad2P in response to TGFβ in different cell lines. Cells were treated with 100pM TGFβ in the presence or absence of the TGFβ receptor I (TBRI) inhibitor SD-093 (1uM; Scios, Inc) then lysed and subjected to SDS PAGE. Transferred proteins were detected with antibodies to the phosphorylated form of Smad-2 (Smad2P) or the unmodified form of the protein (Smad2). Lanes labeled 1029 refer to D6 cells **C:** E-cadherin localization in different cell lines treated or not with 100pM TGFβ. E-cadherin is visualized as red fluorescence. Black arrows indicate cells with E-cadherin localized to the membrane; white arrowheads indicate cells with diffuse E-cadherin staining. Original magnification 400×.

Figure 2.

EMT occurs *in vivo* in choroidal metastases. Metastatic lesions in the choroid of eye arise in mice injected with r3T cells. These tumors were fixed and sectioned and immunohistochemistry was performed for vimentin (Vim), cytokeratin 18 (CK18) and phosphorylated Smad-2 (Smad2P). Sections were counterstained with hematoxylin. The brown color indicates positive staining. Sections incubated with (left side panels) or without (right side) primary antibody are shown. Choroidal melanocytes that are integrated throughout the tumor are intensely brown in some control sections. Original magnification, 252 $\times$.

Figure 3.

A: Vimentin expression in different epithelial cell lines treated with TGF β . Cells were plated in complete medium at time 0 and 100pM TGF β was added at different times after plating. Cells were harvested at 144 hours, and RNA was prepared and subjected to northern blotting as described in methods. The cell line is indicated above the lanes. Lanes 0: 0 hours; 1: 24 hours; 2: 48 hours; 3: 72 hours; 4: 96 hours; 5: 144 hours exposure to TGF β . VIM- human vimentin probe; GAPDH – loading control. **B:** Quantitation of the data from Figure 3A. Background corrected vimentin expression was normalized to GAPDH, and expressed as a relative value. **C:** Western blot of vimentin protein in different cell lines. Cells were treated (+) or not (–) with 100 pM TGF β for 6 days, and protein extracts separated by SDS PAGE. Top panel was reacted with a vimentin antibody, and the 60 kD vimentin band is indicated by an arrow; the lower panel was reacted with an antibody to β -actin. **D:** Quantitative PCR of basal vimentin mRNA levels in different cell lines. Cells were harvested when 80% confluent, and vimentin RNA assessed by quantitative PCR and normalized to β 2 microglobulin. Each point represents the average of duplicate determinations. **E:** Quantitative PCR of vimentin

expression in different *ras*-transformed cell lines/clones. Mouse vimentin values were normalized to GAPDH expression. Values are mean, SD; n=2.

Figure 4.

A: Cytokeratin 18 expression in different cell lines in the presence and absence of TGF β treatment. Northern blot of different cell lines as indicated above the lanes; Cells were treated or not with TGF β (100 pM) for 6 days. CK18 – cytokeratin18, GAPDH, loading control. **B:** Western blot analysis of triton X-100 soluble protein from cell lines as indicated with (+) and without (-) TGF β treatment for 6 days. N- NIH3T3 cells. Arrow indicates the position of the cytokeratin band at 50Kd.

Figure 5.

Analysis of vimentin promoter activity in r3, r3T and NIH3T3 cells. **A:** successively smaller fragments of the vimentin promoter fused to chloramphenicol acetyl transferase (CAT) were transfected into r3 or NIH3T3 cells. The next day, CAT activity was determined and was normalized to a cotransfected luciferase construct. Control is pGL3 control (Promega), a constitutively active promoter driving luciferase. Results are representative of two independent experiments. pGL3 – positive control. **B:** A 4000 nucleotide fragment of the vimentin upstream sequence and the 411 nucleotide sequence were cloned upstream of firefly luciferase. Cells were cotransfected with these plasmids and with renilla luciferase for normalization. The relative expression of the two plasmids (firefly luciferase/renilla) in the NIH3T3 (3T3), r3 and r3T cells is shown. **C:** Relative expression of the -4000 and the -411 plasmid in the three different cell lines. In each case, the normalized firefly luciferase activity from the -4000 construct is expressed as a percentage of the activity of the -411 construct. Mean and SD are shown; n=3. **D:** Effect of TGFβ on the promoter activity of the different constructs. Normalized firefly luciferase activity from the -411 and -4000 promoters was determined in r3T cells treated or not with 100pM TGFβ for 9 days. T- TGFβ

Figure 6.

A. Vimentin mRNA level is not affected by 5-azacytidine. r3T or NIH3T3 cells were plated in 6 well plates in the presence or absence of 10 μ M 5-azacytidine. Cells were harvested at the indicated times after addition of 5-azacytidine (control cells were harvested on day 2), and vimentin mRNA level determined by qPCR, and normalized to GAPDH. **B.** Half life of vimentin mRNA is similar in NIH3T3 and r3T cells. NIH3T3 and r3T cells were plated in 6-well plates and treated with vehicle (0 Hr) or 100 μ M DRB for 3, 6 or 9 hours. At the appropriate times, RNA was harvested, and vimentin mRNA was quantified by qPCR and normalized to GAPDH mRNA levels.