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Review Article

PAI-1 is a Critical Upstream Regulator of the TGF-β1/EGF-Induced Invasive Phenotype in Mutant p53 Human Cutaneous Squamous Cell Carcinoma

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The emergence of highly aggressive subtypes of human cutaneous squamous cell carcinoma (SCC) often reflects increased autocrine/paracrine TGF-β synthesis and epidermal growth factor receptor (EGFR) amplification. Cooperative TGF-β/EGFR signaling promotes cell migration and induces expression of both proteases and protease inhibitors that regulate stromal remodeling resulting in acquisition of an invasive phenotype. TGF-β1+EGF stimulation increases the production of several matrix metalloproteinases (MMPs) in human SCC. Among the most prominent is MMP-10 which is known to be elevated in SCC in situ. Activation of stromal plasminogen appears to be critical in triggering downstream MMP activity. Paradoxically, PAI-1, the major physiological inhibitor of plasmin generation, is also up-regulated under these conditions and is an early event in progression of incipient epidermal SCC. A model is proposed in which TGF-β1+EGF-dependent MMP-10 elevation directs focalized matrix remodeling events that promote epithelial cell plasticity and tissue invasion. Increased PAI-1 expression serves to temporally and spatially modulate plasmin-initiated pericellular proteolysis, further facilitating epithelial invasive potential. Defining the complex signaling mechanisms that maintain this elegant balance is critical to developing potential therapeutics for the treatment of human cutaneous malignancies.

1. HUMAN EPITHELIAL SKIN CANCER PROGRESSION

Cutaneous cancer is the most common human malignant disease [1]; in North America alone, >50% of all neoplasms arise in the skin [2]. The development and progression of epithelial skin tumors is causally linked to ultraviolet (UV) radiation exposure, with UV-B "signature" base changes (C→T or CC→TT) frequently mapping to codons 177 (basal cell carcinoma) and 278 (squamous cell carcinoma (SCC)) in the tumor suppressor p53 gene [3, 4]. Indeed, UV-associated p53 mutations regularly occur in the solar radiation-induced premalignancy actinic keratosis. Approximately 10% of these precancerous lesions progress to SCC and it has been estimated that 60% of all SCC arise within actinic keratoses [4–6].

The progression sequence for cutaneous cancers may vary between the human disease and its corresponding mouse models, although several genetic events are common to both [2, 3, 5–7]. Transition of a normal keratinocyte to an initiated pre- or early malignant phenotype for example often involves p53 inactivation, *ras* gene mutation and amplified *ras* expression. These changes frequently accompany growth of lesional subsets in both actinic keratosis and SCC [5–7]. Recent findings suggest that the emergence of highly aggressive subtypes of SCC (including the lethal spindle cell tumor) and the development of metastatic variants are causally linked to overexpression of transforming growth factor-β1 (TGF-β1) [2, 8–10]. Elevated autocrine and/or paracrine production of TGF-β1, in fact, typifies advanced pathologies in both mouse and human SCC [8, 10]. Despite high levels of TGF-β in the immediate tumor microenvironment, at least some malignant epithelial cells become refractory to the normal program of proliferative arrest initiated by TGF-β which is likely a consequence of

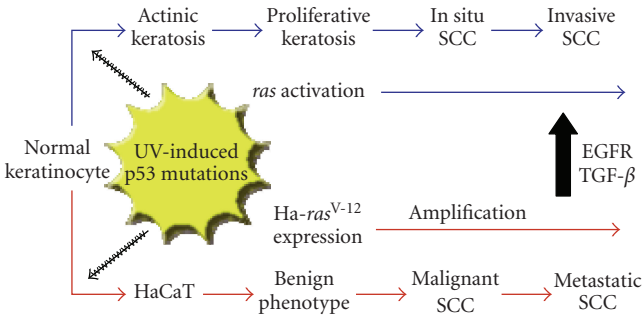

Figure 1: Genetic events associated with human cutaneous SCC progression in vivo and in the HaCaT keratinocyte model system in vitro. Additional similarities are discussed in the text as well as in [2, 3, 21].

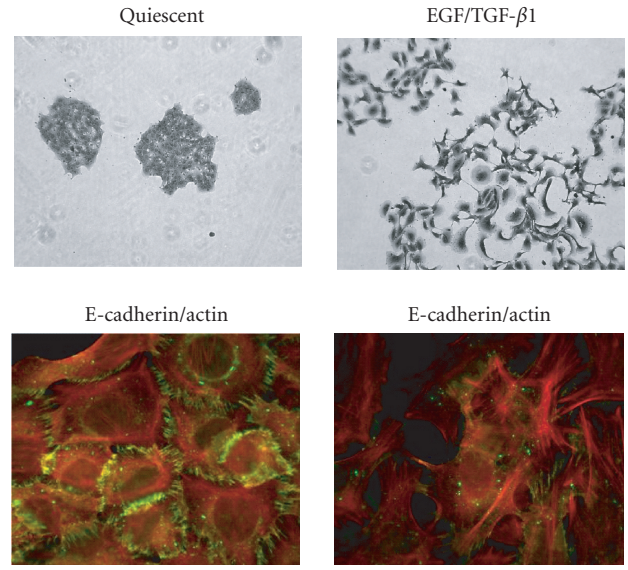

Figure 2: HaCaT-II4 keratinocytes initiate a prominent "scattering" response after a 24–48 hour exposure to EGF/TGF-β1. Colony dispersal (top panels) reflects the early and significant loss of E-cadherin-positive cell-cell junctions (green) and marked reorganization in the actin microfilament system (red) (bottom panels). Such morphologic restructuring is a hallmark of epithelial plasticity initiated by TGF-β and EGF family members.

transformation-associated reductions in either TGF-β-RII or Smad-4 levels, or both [10–12]. In experimental models of skin carcinogenesis, moreover, resistance to TGF-β1-induced growth suppression is often coupled with epidermal growth factor receptor (EGFR) amplification, particularly during the later stages of tumor progression [13–17]. Indeed, cutaneous SCCs frequently exhibit constitutive activation of the EGFR as a result of receptor amplification and/or autocrine ligand release [18]. The subsequent reprogramming of gene expression in the transformed keratinocyte initiates and perpetuates the TGF-β1-induced pro-oncogenic switch to a "plastic" phenotype, resulting in the transition from a relatively indolent to a highly aggressive and invasive epithelial malignancy [8, 19, 20].

2. DETERMINANTS OF CELLULAR PLASTICITY IN TRANSFORMED HUMAN KERATINOCYTES

The immortalized adult human keratinocyte cell line HaCaT-II4 is particularly suited for assessment of molecular mechanisms associated with epithelial tumor cell plasticity (reviewed in [13]). HaCaT-II4 cells harbor mutations that mirror those associated with cutaneous malignant transformation. These include UV-specific mutations in both alleles of the p53 gene (resulting in loss of p53 function [3]), increased levels of an activated Ha-*ras* gene, and chromosomal aberrations often typical of SCC (e.g., loss of 3p and 9p, gain of 3q) [2, 3, 21] (Figure 1).

HaCaT-II4 cell stimulation with a combination of TGF-β1 and EGF, designed to mimic the elevated TGF-β1 expression/amplified EGFR signaling that frequently accompanies SCC progression in vivo, promotes a phenotypic transition that involves the loss of E-cadherin from cell-cell junctions, actin microfilament remodeling (Figure 2), increased motility, and significantly enhanced pericellular proteolytic capability [22, 23].

Stromal proteolysis by transformed keratinocytes is often initiated by conversion of epidermal matrix plasminogen to the broad-spectrum protease plasmin via urokinase plasminogen activator receptor (uPAR)-bound uPA [24–26]. Plasmin generation accompanies cooperative TGF-β/EGFR signaling during epidermal tumor progression and appears to be a critical event in the downstream activation of a complex and highly interdependent, matrix metalloproteinase (MMP) cascade (reviewed in [23]). Microarray profiling of HaCaT-II4 cells stimulated with both TGF-β1 and EGF confirmed, in fact, that uPA, uPAR, and MMP expression levels were significantly upregulated (e.g., Figure 3). Transcripts encoding plasminogen activator inhibitor type-1 (PAI-1; SERPINE1), the major physiological regulator of plasmin-based pericellular proteolysis, were also significantly increased. Indeed, elevated PAI-1 tumor levels signal a poor prognosis and reduced disease-free survival in patients with breast, lung, ovarian, and oral SCC [26, 27]. Mouse modeling and genetic studies clearly implicate PAI-1 as an important determinant in cutaneous tumor invasion and the associated angiogenic response. This serine protease inhibitor maintains an angiogenic "scaffold," stabilizes nascent capillary vessel structure, and regulates tumor cell invasion through precise regulation of the peritumor proteolytic microenvironment [26, 28–30]. PAI-1 upregulation is, in fact, an early event in the progression of incipient epidermal SCC, where it often localizes in tumor cells and myofibroblasts at the invasive front (Figure 4), and most importantly is a tumor marker with significant prognostic value [27, 31–33]. Furthermore, identification of PAI-1 in SCC-proximal stromal

myofibroblasts implies a more global involvement in modulating cellular invasive potential, [34–36] with complex autocrine and paracrine loops dictating the varied effects of this SERPIN on individual elements (neoplastic, endothelial, and inflammatory cells) within the tumor microenvironment.

3. GROWTH FACTOR-INITIATED EPITHELIAL PLASTICITY ELICITS A PROGRAM OF MATRIX REMODELING

Treatment of HaCaT-II4 cells with TGF-β1 and EGF promotes a plastic transition typical of late-stage SCC progression (Figure 2). Part of this response most likely reflects the transcriptional consequences associated with deregulated growth factor signaling (e.g., Figure 3) [37–40]. TGF-β1 stimulates synthesis of stromal components (e.g., fibronectin, collagen, laminin), thereby supporting the maintenance of matrix integrity; this growth factor, however, also increases expression of several extracellular matrix-degrading MMPs, including MMP-1, -2, -3, -9, -10, -11, -13, and 21 [41–47]. Unlike the normal epithelium, where TGF-β1 upregulates collagen synthesis and represses collagenase proteolysis, TGF-β1 usually decreases collagen synthesis and induces collagenase activity in malignant cells, suggesting that transformed epithelia exhibit an altered response to TGF-β1 [48–51]. EGF stimulation similarly induces expression of several MMPs [52–54]. Consequently, a TGF-β1-enriched tumor microenvironment coupled with amplified EGFR levels and/or signaling correlates strongly with the increased expression of MMP-2, -7, -9, 10, -11, and -13 [17, 55] and is frequently associated with advanced pathological stages in human SCC. The expression of MMP-10 (stromelysin-2) following costimulation of HaCat-II4 cells with TGF-β1 and EGF is particularly significant [22, 23]. MMP-10 is generally restricted to epithelial cells [46, 56] and has broad substrate specificity, including as targets the proMMPs-1, -7, -8, -9, and -13, collagens types III, IV, and V, gelatin, elastin, fibronectin, proteoglycans, and laminin [25, 57]. MMP-10 is not detectable in normal intact skin [46]. It is however, expressed during cutaneous injury repair where it localizes to migrating keratinocytes at the wound edge, suggesting that MMP-10 facilitates invasive behavior [46]. Indeed, appreciable levels of MMP-10 are evident in SCC of the head and neck, esophagus, oral cavity, and skin, as well as in recurrences of nonsmall cell lung cancer where it likely regulates basement membrane degradation and stromal dissemination [55, 58–64]. Notably, TGF-β1/EGF-dependent upregulation of MMP-10 in HaCaT-II4 cells is coincident with enhanced collagen gel invasion (Figure 5) and the development of an acute collagenolytic phenotype that is sensitive to components of the plasminogen activation system, including PAI-1 [22, 23]. While the actual involvement of MMP-10 in late-stage tumor progression remains to be clarified, MMP-10 can "superactivate" collagenase I (MMP-1) resulting in a 10-fold increase in specific activity when compared to MMP-1 activation by plasmin alone [56]. Collectively, these findings support a model in which TGF-β1/EGF-initiated MMP-10 upregulation and its plasmin-dependent activation lead to the degradation of extracellular matrix components directly, as well as indirectly by its ability to trigger MMPs-1, -7, -8, -9, and -13 activities (Figure 6). Subsequently, these downstream proteases target stromal substrates, particularly collagens and additional pro-MMPs in the tumor microenvironment. The resultant feedback loop generated through elevation of MMP-10 levels therefore supports focalized extracellular matrix remodeling which promotes the acquisition of cellular plasticity and tumor cell invasion. Most importantly, this highly interactive plasmin-initiated, pericellular proteolytic cascade is finely "titrated" both temporally and spatially by PAI-1, highlighting the potential therapeutic value of manipulating PAI-1 expression in the treatment of human cutaneous malignancies [13, 22, 23, 29, 30, 65].

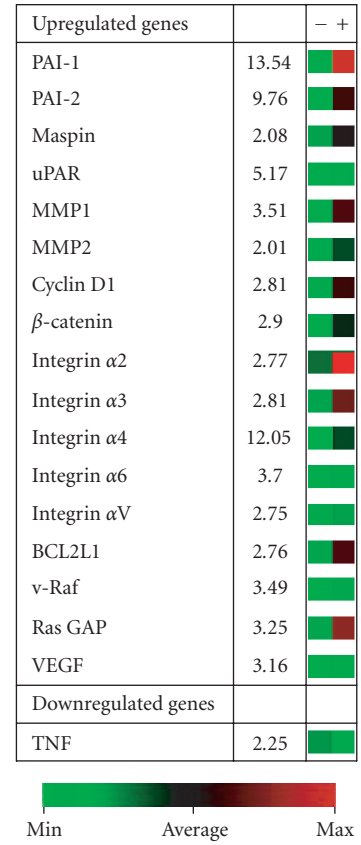

Figure 3: Example of a selected cluster of TGF-β1+EGF-induced genes in HaCaT-II4 human SCC cells. PAI-1 is the highest upregulated transcript in the subset illustrated. (13.4-fold assessed 6 hours after growth factor stimulation). MMP-1 and MMP-2 are also significantly increased in response to TGF-β1+EGF as is the urokinase plasminogen activator receptor (uPAR). The 5-fold induction of uPA mRNA is not shown. Numbers for the individual upregulated expressed genes indicate the fold increase for TGF-β1+EGF-stimulated cells compared to unstimulated keratinocytes. The colorized platform serves to provide a visual indicator of the microarray data with green signal corresponding to minimal or nonexpressing status while red signal is indicative of high-level transcript induction.

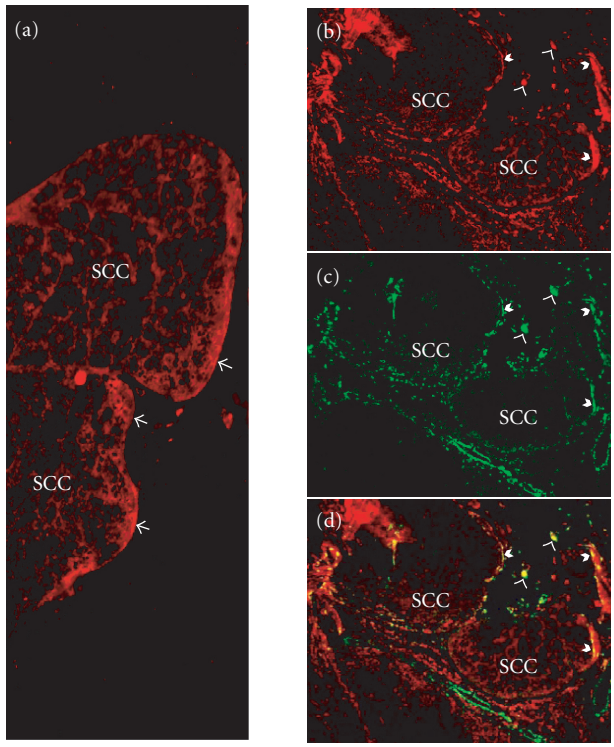

Figure 4: Sections of an early invasive human squamous cell carcinoma (SCC) were stained for PAI-1 (red) and α-smooth muscle actin (green). (a) Demonstrates the localization of PAI-1 at the invasive front of the tumor (arrows). (b) (PAI-1), (c) (α-smooth muscle actin), and (d) (merged) illustrate the colocalization of PAI-1 with cells stained positive for α-smooth muscle actin, a marker for myofibroblasts. Barbed arrows indicate PAI-1/α-SMA at the tumor perimeter, while arrow heads depict PAI-1/α-SMA in the stroma.

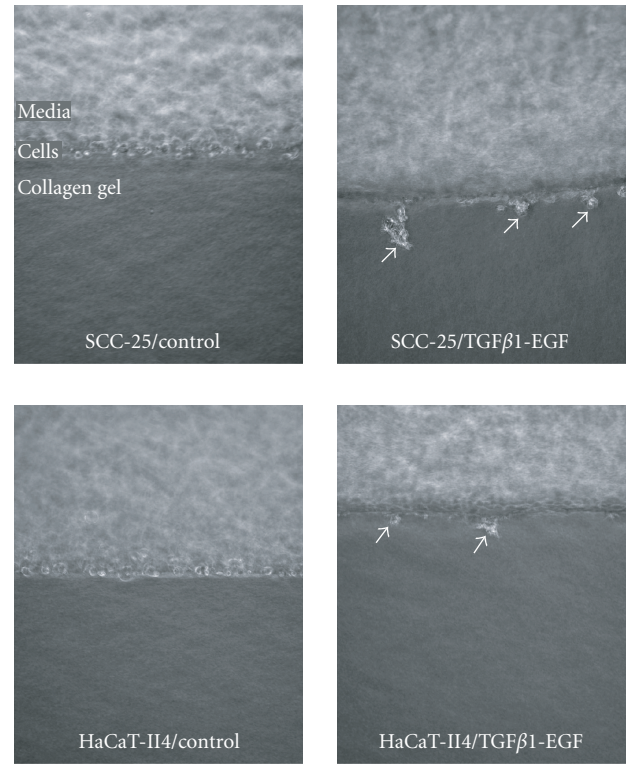

Figure 5: HaCaT-II4 cells invade collagen gels following costimulation with TGF-β1 and EGF. HaCaT-II4 or SCC-25 cells were seeded in serum-free advanced DMEM (GIBCO) onto collagen gels that had been polymerized in OptiCell tissue culture chambers. Twenty four hours later, cells were stimulated with a combination of TGF-β1 (1 ng/mL) and EGF (10 ng/mL) under serum-free conditions and allowed to incubate for 48 hours. Pictures were taken at X10 magnification using an IX70 Olympus microscope and ImagePro-Plus software.

4. TGF-β1/EGFR PATHWAY INTEGRATION IN PAI-1 EXPRESSION CONTROL

Recent studies revealed a more complicated, cooperative interaction between intracellular events orchestrated by TGF-β1-activated pathways and the EGFR, which specifically lead to epithelial tumor plasticity. PAI-1 induction in response to TGF-β1 involves a complex network of signaling intermediates and requires the activities of the mitogen-activated extracellular kinase (MEK), $p21^{ras}$, and $pp60^{c\text{-}src}$ in addition to the EGFR [66]. $pp60^{c\text{-}src}$ is, in fact, a critical intermediate in a TGF-β1-initiated transduction cascade leading to MEK signaling, PAI-1 transcription, and subsequent phenotypic responses [66–70] (Figure 7). The *src* family kinase inhibitor PP1 and dominant-negative $pp60^{c\text{-}src}$ constructs effectively attenuate TGF-β1-induced PAI-1 expression in HaCaT cells [66], confirming the generality of *src* kinase involvement in PAI-1 gene regulation. While the actual mechanism underlying TGF-β1-associated $pp60^{c\text{-}src}$ kinase stimulation remains to be determined, the TGF-β1-dependent release of EGFR ligands HB-EGF and/or TGF-α appears to involve MMP-directed cleavage of EGF-like precursors resulting in EGFR activation [71–73]. Alternatively, formation of integrin/FAK/$p130^{cas}$/EGFR complexes in response to TGF-β1 may result in ligand-independent EGFR mobilization and β increased $pp60^{c\text{-}src}$ activity [74–76]. Subsequent changes in gene programming likely reflect the particular *src*-dependent MAP kinase pathways impacted. *src* kinases, for example, can phosphorylate the *raf*-1 kinase either directly or as part of a CNK1 scaffold complex, resulting in *src*-dependent ERK activation [77–79]. Indeed, the effective blockade of TGF-β1-stimulated ERK1/2 phosphorylation and PAI-1 transcription by PP1 as well as the EGFR inhibitor AG1478 (Figure 7) and the requirement for MEK-ERK signaling for the full inductive effect of TGF-β1, suggests that $pp60^{c\text{-}src}$ may regulate MEK-ERK-dependent PAI-1 expression via EGFR activation at the Y845 site [66, 67, 75].

The continued definition of specific molecular mechanisms underlying control of tumor progression genes is an essential element in the ultimate design of targeted, clinically

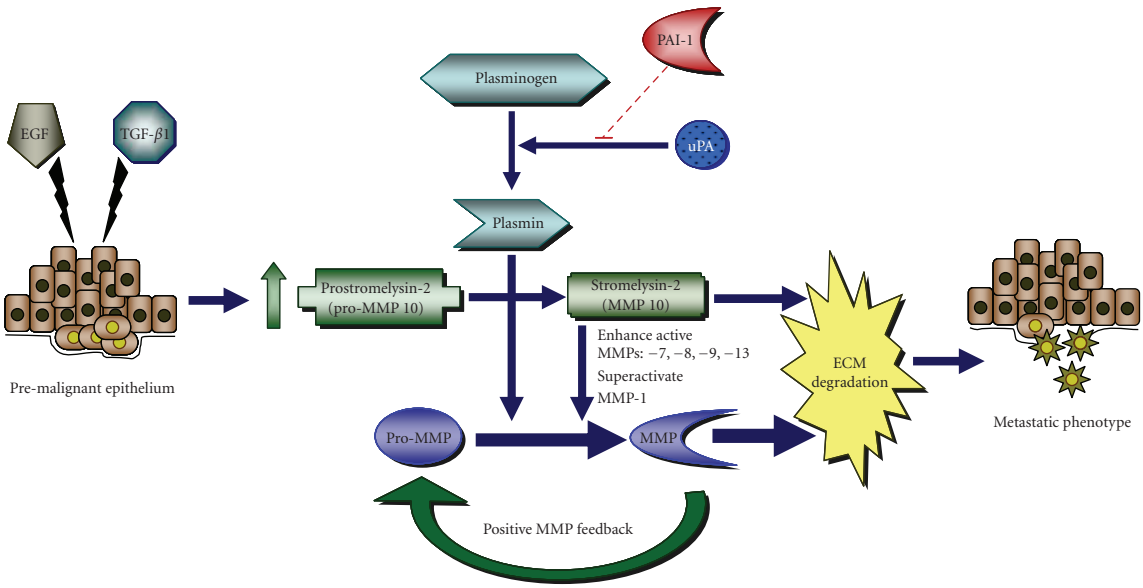

Figure 6: Proposed model illustrating the potential effects of TGF-β1/EGF stimulated upregulation of MMP-10 and PAI-1 on premalignant epithelial cells. (described in text).

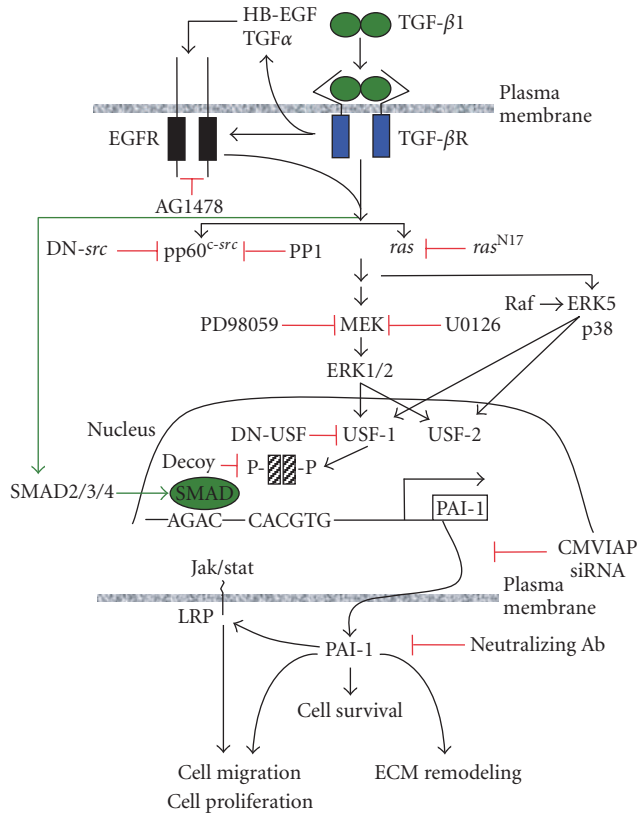

Figure 7: The PAI-1 expression control network. TGF-β1 can signal alone to MEK as well as transactivate the EGFR. This cascade requires the participation of pp60$^{c\text{-}src}$ and *ras*. The downstream-activated MAP kinases (ERKs, p38) phosphorylate, and thereby, regulate the activity of specific transcription factors (e.g., members of the USF family) that are known to impact PAI-1 gene control [13]. PAI-1 expression, in turn, affects cell survival, migration, and matrix remodeling as part of the program of epithelial plasticity. Inhibitors of PAI-1 expression or function are shown in red and represent potential therapeutic target points.

relevant, options for treatment of human cutaneous SCC. Indeed, the emerging appreciation that cooperative EGFR signaling is an essential aspect of TGF-β1-stimulated PAI-1 expression provides novel insights to the impact of TGF-β1 in late-stage human tumor progression and underscores the potential diversity of new molecular targets that can be exploited for therapeutic benefit. Refining the current understanding of PAI-1 gene regulation, as well as its signaling pathways, may lead to the design of transcription-focused "therapeutics" to manage human cutaneous malignancies.

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