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The Conundrum on the Role of Transforming Growth Factor Beta Superfamily with Reference to Follistatin Related Gene FSTL3 in Breast Cancer Metastasis

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Abstract

Carcinogenesis is fundamentally the result of abnormalities in cell division functions, leading to unabated proliferation of cells. The key to finding a "cure" for all forms of cancer is to understand the fundamental genetic mechanisms of lack of cell cycle control, and to understand the genetics of immune response (how tumor escapes the immune surveillance) towards cancer cells so that one can start developing effective immune-based therapeutics such as the currently popular immune-checkpoint therapeutics. This review focuses on the role of Transforming Growth Factor-Beta (TGF-β) signaling pathways in molecular pathogenesis is of cancer along with possibility of finding novel biomarkers and targets for novel therapeutics. TGF-β is a versatile cytokine and it is a key player in normal development as well as Tumor development. It is a pleotropic cytokine with varied effects on varied cells and certainly has influence on cellular transformation (such as epithelial to mesenchymal transition) invasion, regulation of immune responses (such as T cell responses, and microenvironment modification that cancer cells may exploit to dislodge and migrate (metastasize). Hence, the TGF-β response is pertinent with respect to development and normal functions of the body and also in modulating carcinogenesis. Hence, unravelling the effects of TGF-β signaling pathway could lead to the in-depth understanding of its basic molecular role in triggering or transforming normal cells to cancerous cells directly or indirectly. Deciphering the molecular mechanisms of TGF-β based initiation process of carcinogenesis could help us to find novel targets for future more effective therapeutic intervention before the tumor becomes established and or metastasized. In the context on the role of TGF-β and carcinogenesis, Follistatin-like 3 (FSTL3) plays an important role. Because FSTL3 binds and inactivates Activin A, a member of the TGF family of proteins. Activin A has high levels of expression in breast cancer cells. Breast cancer cells have been shown to become insensitive to the growth-inhibiting action of Activin. This loss of sensitivity is caused partly by the expression of follistatin, and follistatin-related genes (FLRG) such as FSTL3, which bind to Activin A with high affinity thereby inhibiting Activin A's growth inhibiting function. Although FSTL3 expression levels are varied in human tumors, previous studies have shown that there is an increased presence of FSTL3 in invasive breast cancer tumors. However, it's not known yet whether the FSTL3 expression levels in breast cancer tumors correlate with and are causal to tumor growth and metastasis. Structurally, FSTL3 binds with much stronger affinity to Activin A than to other TGF-β superfamily ligands because of the strong interaction of the N-terminus of the FSTL3 protein with Activin A. So what essentially we are addressing in this issue is if FSTL3 could be a desired biomarker in breast cancer along with other downstream molecules associated with TGF and cell cycle events.

Keywords: Carcinogenesis; TGF-β; FSTL3

Introduction

Genesis of cancer involves somatic mutations resulting in activation of oncogenes or de-activation of tumor suppressor genes within the genome. In effect, these alterations in gene functions in normal cells enable them to acquire specific hallmark traits that convert normal cells to carcinogenic and eventually lead to malignancy. The processes of transition of normal cells to carcinogenic phenotype entails acquiring the abilities for (i) sustained proliferative signaling, (ii) evasion of growth suppression, (iii) resisting cell death by apoptosis, (iv) enabling replicative immortality/inhibiting cellular senescence, (v) inducing angiogenesis and (vi) acquiring invasive ability and (vii) evasion of immune surveillance. While tumorigenesis is genetically/epigenetically determined, tumor progression and metastasis are determined by several components of the immune surveillance system of the body. The host immune response towards tumor development involves several soluble mediators released by the immune associated cells besides the immune cells themselves such as cytokines and chemokines. The ability to dislodge and migrate from primary established tumor to a distant site could be mediated by these soluble mediators by facilitating alterations in cellular morphology, such as epithelial mesenchymal transition (EMT) which is known to be associated with tumor metastasis and aggressiveness. A versatile soluble mediator in this regard is transforming growth factor beta (TGF-β), a pleotropic cytokine with growth regulating and immune mediating potential.

Where does this TGF-β come from in the tumor environment?

In general, TGF-β is expressed by normal cells in normal tissues as growth factor to deal with physiological tissue injuries and

homeostasis. However, under pathological tissue injuries, TGF-β may be secreted by blood platelets and various stromal components to prevent uncontrolled cell proliferation and inflammation. This scenario might occur in tumors as well since TGFβ is generally present in the tumor microenvironment, initially to prevent progression towards malignancy, subsequently as a factor that malignant cells may use to their own advantage in terms of proliferation EMT and invasiveness. The presence of TGF-β has been documented in many subsets of tumors, commonly assayed by Smad2 C-terminal phosphorylation [1].

Tumor progression is characterized by the level of uncontrolled proliferation of cells. Tumors begin as localized monoclonal or polyclonal expansion of cells. As these cells accumulate, this marks the transition from pre-malignant lesions to primary tumors. Tumors become malignant, invasive, and metastatic when cancer cells disseminate from the primary tumors and spread to distant organs through blood vessels and/or lymph vessels.

As tumors progress and metastasize, the chances for patient survival decrease across all types of cancers. For example, the five-year-survival rate for prostate cancer decreases from almost 100% for local stage to 28% for distant stage. Similarly, the five-year-survival rate drops from 49% to less than 1% from Stage IA to Stage IV metastatic disease in non-small-cell lung cancer and from nearly 100% in Stage 0 or 1 to 22% in Stage IV in breast cancer. Staging of any type of cancer represents the degree to which the tumor cells have metastasized from the initial development of primary tumor. The aggressiveness of the tumor is partially judged by the degree of metastatic potential which can be influenced by the host's immune system, particularly by the soluble immune mediators as described above. As tumor cells proliferate at an increasingly aggressive rate, they become accordingly difficult to treat, thus accounting for the decreasing survival rates with later stage cancers. What needs to be tackled is how to avoid the entry of the normal cells into uncontrolled replication and evasion of transformed cells from the immunological surveillance to avoid elimination of such transformed cells by the immune cells.

This review focuses on the issue of uncontrolled replication of normal cells and loss of cell cycle regulation. In this context, we examine the role TGF-β family signaling pathways in molecular pathogenesis are of cancer. TGF-β is a versatile cytokine and it is a key player in normal cell development as well as in tumor cell development. It is a pleotropic cytokine with varied effects on varied cells and certainly has influence on cellular transformation (such as epithelial to mesenchymal transition) invasion, regulation of immune responses (such as T cell responses, and microenvironment modification that cancer cells may exploit to dislodge and migrate (metastasize). Hence, the TGF-β response is pertinent with respect to development and normal functions of the body and also in modulating carcinogenesis. Therefore, it is important to understand the effects of TGF-β signaling pathway to lead towards the in-depth understanding its basic molecular role in triggering or transforming normal cells to cancerous cells directly or indirectly. Unravelling the molecular mechanisms of TGF-β based initiation of processes that lead to carcinogenesis could help us to find novel targets for future more effective therapeutic intervention before the tumor becomes established and or metastasized.

Epithelial Mesenchymal Transition (EMT) and the role of TGF-β signaling

Increased ability of cancer cells to dislodge and metastasize into distant organ confers the degree of aggressiveness to a malignant tumor. Such ability to dislodge and translocate (migrate) is acquired by the transformation of cancer epithelial cells into a mesenchymal phenotype by a process called epithelial mesenchymal transition (EMT). Essentially, EMT facilitates tumor metastasis by directing the development of motile mesenchymal cells from non-motile parent epithelial cells. After invasion, these tumor cells can go back to epithelial phenotype by the reverse process namely mesenchymal epithelial transition (MET) and divide, proliferate and form new tumors. Among the several cytokines and growth factors that favor EMT, TGF-β is the most potent one, and from this perspective enhanced TGF-β signaling could result in increased EMT and increased metastatic potential of the tumor cells. The process of EMT involves loss of E-Cadherin from the epithelial cells and acquiring of mesenchymal cell markers such as Vimentin, α-Smooth Muscle Actin (SMA) among others [2,3].

TGF-β upon binding to its cellular receptors initiates multiple intracellular signaling pathways leading to EMT. In the Smad dependent TGF-β signaling, smad2/3 gets phosphorylated and gets imported to the nucleus. The activation of the TGF-β receptor induces phosphorylation of Smad2/3 proteins, which are then imported into the nucleus with Smad4. Nuclear Smads act as cofactors for transcriptional proteins such as lymphoid enhancing factor 1/T-cell factor–β-catenin complex. The activation of β-catenin is thought to induce EMT [4]. Other transcription such as Twist, fibroblast transcription site-1–binding proteins are also involved in TGF-β induced EMT. There are also reports on the involvement of p38 MAPK pathways in TGF-β mediated EMT [5].

Potential molecular and biochemical association of TGF-β superfamily with genesis of cancer and metastasis

Loss of regulation of the cell cycle is a key step in the transformation from normal cell to a cancerous one. Cancer cells in a primary tumor continue to evolve through expression of various genes that enable them to survive against the immunological surveillance mechanisms to eliminate such cells. In this evolving process some cancer cells in primary tumor acquire abilities to disseminate from primary tumor mass and migrate to various organs through blood circulation and colonize in those organs, a process known as metastasis. For this reason, genes and their resulting proteins, such as p53, which plays an important regulatory role in the cyclin-CDK activation pathway during the cell cycle, have been of great interest in cancer research.

TGF-β pathway activation results in regulation of transcription of proteins that are important in cell cycle and cell growth, and studies have shown that this pathway exhibits differential behavior during tumorigenesis. TGF-β stimulation could inhibit cancer cell proliferation in some cells and enhance in others. There are several factors that are involved in TGF-β-regulated cell proliferation and keep its signaling pathways balanced. Blocking or inhibition of TGF-β signaling can lead to a loss of balanced TGF-β signaling, resulting in the generation and progression of tumors. TGF-β signaling is mediated by Smad proteins and non-Smad pathways. TGF-β stimulation may inhibit cancer cell proliferation in some cellular contexts and promotes it in some other contexts TGF-β-actions on cells are multifactorial. Hence essentially, TGF-β can promote or inhibit cancer progression

and it appears that it all depend on the cell types, the tumor, and the molecular determinants active in each tumor type that are present in each tumor cell mass.

TGF-β is an established growth factor and cytokine involved in inducing epithelial-mesenchymal transition in mammary and lung cells [6,7]. Based on the role of TGF-β in the induction of EMT an event that triggers migration of tumor cells to distant organs (tumor metastasis) it is fair to assume that TGF-β signaling could be pivotal in controlling or regulating breast cancer metastasis. Anything that can influence TGF-β signaling positively or negatively is likely to have effects on tumor pathogenesis in terms of metastasis and evasion from immune responses. In-depth understanding of all the molecular events (molecular details of the intracellular signaling pathways leading to EM) could open up new avenues for potential targeted therapeutics. It is in this context this review is focusing on FSTL3.

TGF superfamily member Activin A in cancer

Members of TGF-β superfamily perform diverse functions depending on their interaction with different downstream targets. For example, different TGF-β family members specifically activate either types of type II or type I receptors, which in turn phosphorylate and activate different Smad molecules. Thus various TGF-β pathways exhibit diverse functions on cancer progression.

In addition to inducing EMT which favors migration/metastasis of tumor cell, TGF-β can also induce angiogenesis, especially in the later stages of cancer development. Formation of new blood vessels also facilitates the migration and metastasis of tumorous cells. Activin A has been shown to be responsible for cell proliferation of malignant mesothelioma cells. Activin A has also demonstrated effects of growth inhibition in human breast cancer cells. This pathway also acts as a tumor suppressor for gastric cancer, and has shown to be more potent than TGF-β. Hence it appears that while TGF-β might favor tumor metastasis, activin, another member of the TGF-β superfamily, could counteract that action of TGF-β. As we noted before, the ligands that bind TGF-receptors may determine which pathways predominate in the tumor with the ability to determine the subsequent fate of the tumor.

While in breast cancer and prostate cancer activin has tumor suppressive function, in lung and head and neck squamous cell carcinoma, activin A supports tumor cell proliferation and invasion. It is therefore important to understand the activin A signaling in comparison to TGF-β and how activin A favor tumor progression and metastasis in some cancers ad inhibit tumor invasiveness in some other cancers. [8].

What we need to work with are the TGF Superfamily members namely TGF-superfamily Type I/II receptors and the ligands TGF-β, activin A and BMP-7.

Activin A

The role of activins in breast cancer progression is not well studied. Earlier, downregulation of activin signaling in breast tumors has been reported [9]. On the contrary, increased serum level of activin has been reported in women with breast cancers [10].

Bashir et al. reported that activin-A signaling is hyperactivated in breast cancers indicated by higher activin-A, phosphoSMAD2, and phosphoSMAD3 levels in advanced breast cancers. They observed downregulation of bone morphogenetic proteins and indicating its suppression in breast cancers. Activin-A expression correlates inversely with survival and metastasis in advanced breast cancers using microarray analysis was performed to reveal gene expression changes in breast tumors, quantitative PCR and immunohistochemical analysis in two independent sets of normal and tumor samples. These authors used recombinant activin-A, inhibitors, overexpression, and knockdown strategies both *in vitro* and *in vivo*, to determine the mechanism underlying the protumorigenic role of activin mediated signaling [11].

Activin, unlike TGF-β is secreted as an active protein. Upon binding to its receptors ActRII/IIB, similar to TGF-β signaling, SMad2/3 gets phosphorylated and complexes with Smad4 and this complex gets translocated into the nucleus initiating transcription of downstream effector molecules. Activin also can engage in non-canonical signaling such as as Akt/PI3K, MAPK/ERK and Wnt/β-catenin that are independent of Smad based signaling. Essentially while TGF-β and activin share signaling pathways, they also act through distinctive downstream effector molecules [12].

And hence TGF-β mediated and Activin mediated actions could have fundamentally distinct functional roles [13].

So as for the conundrum in cancer, because of the overlapping signaling and independent pathways of activin A and TGF-β, it is unraveling specific downstream transcriptional targets for each of these pathways is rather difficult, more so in adult tissues. Both activin A and TGF-β, *via* are known to be involved in regulation of various cell cycle and extracellular matrix proteins [14]. With current cutting edge molecular technologies, future studies might be able to have in-depth understanding of Activin/TGF-β signaling targets in the same tissues, and in the context of this review in breast cancer tissues, and to unravel process of breast cancer aggressiveness and metastasis.

Although activin and TGF-β share growth suppressive SMAD signaling in colon cancer, they diverge in their SMAD4-independent pro-migratory signaling utilizing distinct mitogenic signaling pathways that affect EMT. p21 localization in colon cancer may determine a dominant activin versus TGFβ ligand signaling phenotype warranting further validation as a therapeutic biomarker prior to targeting TGFβ family receptors [15].

Regulation of Activin A expression by follistatin and inhibin

Several mechanisms are involved in the tight regulation of Activin. Most well studied and best understood are the ones mediated by follistatin and Inhibin A, that are endogenous inhibitors found at the cell membrane and in circulation [16].

The difference in effect between pathways for the same disease, such as their effects on gastric cancer, may be attributed to their main mechanistic differences: particularly the actions of Smad molecules. The Smad molecules are responsible for carrying on downstream regulation of the genes, relatively distant from the site of the type I and type II receptors. The role of the smad molecules in the overall pathway activity offers the possibility that downstream activity may determine activin A and/or TGF-β effects on tumor cells. Another possibility is that activity at the receptor level itself affects regulation of activity of the TGF-β pathways based on the binding ligand, and their subsequent disruptive modification that allows for the abnormal cell activity of malignant tumor growth.

So while TGF-β mediated signaling events may lead towards tumor metastasis through EMT, Activin mediated signaling may curtail the

ability of the tumor metastasis by inhibiting cell proliferation. What we don't know now is what if anything; control the expression of Activin A which is crucial to the further understanding of the Activin mediated effects on tumor cells.

So, as far as we know now, signaling events by TGF superfamily members exerts diverse range of effects on malignant tumors, both depending on the type of tumor as well as the stage of progression. The diversity of this pathway and the prevalence of cancer types that have been linked to abnormalities within the TGF superfamily pathway demonstrate why investigation of the molecular mechanisms of TGF-β-Ligand interactions and down-stream events in cancer research. In order to connect all the dots, first we need to have in-depth understanding of the important dots. To this end, attention is being made towards Follistatin Related Gene namely, *Follistatin-Like 3* (*FSTL3*) in relation to TGF-β superfamily mediated signaling that may promote or inhibit cancer aggressiveness and metastatic potential.

Breast cancer cells have been shown to become insensitive to the growth-inhibiting action of activin, a member of the TGF-β family pathway. This loss of sensitivity has been shown to be partially caused by the expression of follistatin, and follistatin-related genes (FLRG) such as *FSTL3*, which bind to activin with high affinity and inhibit its function. Although FSTL3 show variable expression level in human tumors, previous studies have shown that there is an increased presence of FSTL3 in invasive breast cancer tumors. However, it's not known yet whether the FSTL3 expression levels in breast cancer tumors correlate with and are causal to tumor growth and metastasis.

If a definitively correlative and eventually causal relationship could be observed between increased FSTL3 presence and increased breast cancer metastasis, we can develop specific small molecular inhibitors to block FSTL3-mediated tumor progression, increasing patient survival. In addition, increased levels of FSTL3 in circulation and tumor could be used as a prognostic biomarker.

Follistatin related gene: FSTL3

Follistatin-like 3 (FSTL3) binds and inactivates activin, a member of the TGF family of proteins and a growth factor involved with cell growth and differentiation. Activin A exhibits high levels of expression in breast cancer cells. Structurally, FSTL3 binds with much stronger affinity to activin A than to other TGF-B superfamily ligands or bone-morphogenic proteins (BMP) because of the strong interaction of the N-terminus of the FSTL3 protein with activin A. Hence FSTL3 seems to be a desired biomarker in breast cancer.

Studies reported by Pasquapina et al. show that activin A is a differentiation factor of human endometrium, and that their findings support the fact that an imbalance between increased activin A and decreased FLRG expression in endometrial cancer may be pivotal in endometrial tumorigenesis. And this could be the case with breast cancer and metastasis as well [17].

Tsuchida et al. characterized a new follistatin-like protein from mouse consisting of a 256-residue precursor and, a potential mouse homologue of human FLRG. These authors showed that the recombinant mouse FLRG proteins could bind to both activin and bone morphogenetic protein-2 (BMP-2) with higher affinity for activin than for BMP-2. The FLRG protein inhibited activin-induced and BMP-2-induced transcriptional responses in a dose-dependent manner. The implication of his study is that finding cellular and molecular signaling by activin and BMPs are regulated by multiple members of the Follistatin family [18].

Bloise et al. showed that follistatin and FLRG were expressed both in normal tissue as well as in the breast diseases namely florid hyperplasia without atypia, fibroadenoma, ductal carcinoma *in situ* (DCIS) and infiltrating ductal carcinoma (IDC) they investigated investigated. These authors detected follistatin in the epithelial cytoplasm and nucleus in normal, benign and malignant breast tissue, at both mRNA and protein levels. However, FLRG area and intensity of mRNA and protein staining were higher in the cytoplasm and in the nucleus of IDC epithelial cells as opposed to normal breast (NB). They concluded that the up regulation of FLRG in IDC suggests a role for this protein in the progression of breast tumor malignancy. As per their findings, while activin displays an anti-proliferative effect in human breast cells, increased FST and FLRG expression in breast proliferative diseases could counteract the anti-proliferative effects of activin [19].

Razanajaon et al. showed that silencing FLRG induced growth inhibition, which is reversible upon addition of exogenous FLRG. Their study also suggests that FLRG silencing effects resulted from restoration of endogenous activin functions. Interestingly they found that the growth inhibition induced by FLRG silencing was reversible by treatment with a soluble form of type II activin receptor. These authors also observed strong expression of FLRG in invasive breast carcinomas in contrast with the normal luminal epithelial cells concluding that endogenous FLRG contributes to tumor cell proliferation through antagonizing endogenous activin effects [20].

There are several angles of approach to take in order to examine the role of FSTL3 in tumorigenesis. One is to investigate the activity of FSTL3 at the transcription and translation level itself; that is, to determine whether there is an increase in FSTL3 protein levels that is responsible for increased activin inhibition in breast cancer cells. Another is to determine the location of FSTL3 proteins, and whether it acts extracellularly *via* secretion and paracrine activity, or whether this is localized and intracellular activity. The role, or lack thereof, of FSTL3 in other cancer types is also worth looking into, in order to gain more cases to examine.

HeLa human cervical adenocarcinoma cells had previously been shown by to have relatively high level of FSTL3 expression and FSTL3 was previously able to have been knocked down using shRNA. Thus, we chose to knock down FSTL3 in in HeLa cells, which were then injected into athymic nude mice and NSG-SM3 mice. All mice were injected with 50,000 luciferase-labelled LM2 mammary adenocarcinoma cells, plus one million of the shFSTL3 HeLa or control HeLa cells. Luciferase activity was imaged and measured for the mice to indicate tumor cell activity.

From Figure 1, FSTL3 knock down did appear to correlate with a decrease in breast tumor activity in the athymic mice; the trendline for the FSTL3 knockdown mice luciferase activity is less steep than the control mice luciferase activity.

While this is just a limited contrived animal experimental model, it points towards further research to manipulate FSTL3 expression in breast cancer cells to prevent or reduce metastasis.

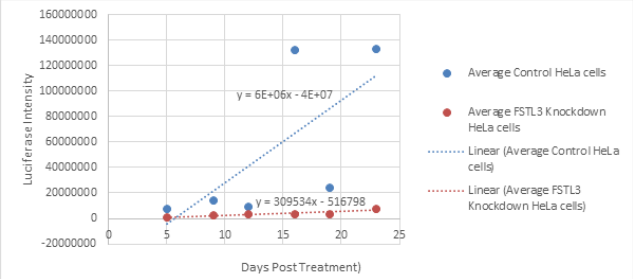

Figure 1: FSTL3 knock down did appear to correlate with a decrease in breast tumor activity in the athymic mice; the trendline for the FSTL3 knockdown mice luciferase activity is less steep than the control mice luciferase activity.

TGF superfamily effects on cell cycle and proliferation, EMT, MHC expression, tumor immune escape

Loss or reduction of MHC class I molecules enables the tumor cells to escape the cytotoxic effects of T cells. Whether the EMT process initiated by TGF-β is mechanistically associated with loss or significant reduction of MHC class I molecules from the cell surface is an area worth studying. EMT, primarily type III EMT, is central to aggressive tumoral phenotypes in lung cancer and has been shown to be associated with poor prognosis [21]. Recent studies have suggested EMT mechanisms to be involved in the immune escape of cancer cells *via* multiple routes, one being the shaping of the tumor microenvironment [22]. However, very little is known regarding the interplay of EMT with immune check point dysregulation and how potential targets could be rationally selected to improve treatment response for emerging conflicts of success in immunotherapy.

Potential relationship between EMT and HLA/MHC expression

Immunoproteasome can generate peptides with high specificity for binding to MHC class I molecules, to induce CD8 T cell mediated cytotoxic responses. Currently, the expression of the immunoproteasome and its influence on tumors of epithelial origin is not well understood. These authors investigated the constitutive and induced expression patterns of immunoproteasome subunits in non-small cell lung cancer (NSCLC) and their consequence on antigen presentation. They observed a highly variable immunoproteasome expression among NSCLC tissues. This could be potentially related to inability to express the appropriate peptides in the context of appropriate MHC/HLA [23].

Why the conundrum? How to delineate and use the signaling events mediated by TGF superfamily members towards better understanding of tumor metastasis?

As described in above sections, while TGF-β can cause EMT, and favor tumor metastasis and angiogenesis, other members such as activin A can inhibit the carcinogenic potential of TGF-β and FSTL3 can bind to activin and prevent its action. Hence, regulation of expression of these TGF family members in tumor microenvironment could be complicated phenomenon.

Conclusion

The potential implications of detecting a correlative and/or causal relationship between FSTL3 presence and cancer development could be significant. The TGF-β pathway and its various ligands, like activin A, have been extensively investigated. However, elucidation of the exact pathway like that discussed here which could lead to play crucial role in the development of cancers is highly variable. This suggests the need to focus on specific molecules that can impact this pathway. FSTL3 is one such molecule that thus far has not been looked into very closely in terms of its exact role in tumorigenesis, yet its high affinity for activin A has been shown by several studies. Cancer treatment and therapeutics research has moved towards personalized and specifically targeted treatment methods in recent years in order to provide an alternative to the current more generalized and often harmful radiation and chemotherapy treatments. Showing a correlation or causal role of FSTL3 in the development of breast cancer would provide a specific target protein that could be used to develop targeted treatment methods.

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