

NFAT in Pancreatic Carcinogenesis

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Abstract

Pancreatic Ductal Adenocarcinoma (PDAC) belongs to the most aggressive malignancies among solid tumors. The increasing incidence of PDAC and its dismal prognosis with a five-years overall survival rate of less than five percent characterize PDAC as one of the most clinically challenging diseases among all malignancies. In recent years, increasing knowledge about the molecular pathogenesis of PDAC has shown that mutational activation of oncogenic Kras represents the defining event in pancreatic cancer initiation, but full neoplastic pancreatic cancer progression requires dysregulation of additional pathways. In this review we summarize and discuss the impact of inflammation-induced transcription of the Nuclear Factor of Activates T-cell (NFAT)-family in pancreatic carcinogenesis.

Keywords: NFAT; Pancreatic cancer; Transcription

Introduction

The current model of pancreatic carcinogenesis describes a stepwise transformation of healthy exocrine cells into highly invasive pancreatic cancer cells [1,2]. Despite the ductal morphology lineage tracing studies revealed that PDAC tumors arise from the acinar compartment. In fact, early genetic alterations in acinar cells induce a transdifferentiation program termed Acinar-To-Ductal Metaplasia (ADM) in which acinar cells adopt a duct-like structure and loose expression of acinar-cell differentiation markers [3]. Depending on the molecular context, ADM lesions then convert to pancreatic cancer Precursor Lesions (PanIN) with increasing degrees of dysplasia and can progress to invasively growing pancreatic cancer [4,5]. The morphological changes during pancreatic carcinogenesis are accompanied by a recurrent pattern of genetic and epigenetic alterations; the earliest and most prevalent of which is oncogenic activation of Kras [1]. Kras-proteins comprise a family of signal-transducing GTPases involved in regulation of vital cellular functions including differentiation, proliferation, survival and apoptosis. Mutational activation of Kras is found in early tumor stages and in virtually all invasively growing pancreatic tumors [6]. The impact of Kras mutation for pancreatic carcinogenesis has been elegantly demonstrated in Genetically Engineered Mouse Models (GEMM) with pancreas-specific constitutive activation of the enzyme (KrasG12D) [5]. However, studies in mice and humans have revealed that Kras mutation alone is not sufficient for full neoplastic progression and rather induces a permanent cell growth arrest called senescence. Therefore, additional genetic and epigenetic events [7] such as deficiency of prominent tumor-suppressor genes, e.g. p53 and p16Ink4a, are required to bypass oncogene-induced senescence and apoptosis and to stimulate tumor promotion and progression [7,8]. Moreover, signals from an oncogenic tumor environment are involved in pancreatic tumor development and progression. This type of pro-oncogenic microenvironment can be provided by sustained organ inflammation, e.g. chronic pancreatitis, which constitutes the most relevant risk factor for PDAC development in human [9,10]. Chronic

pancreatitis supports the initiation and progression of this malignancy by direct modification of gene expression networks in pancreatic epithelial cells, specifically through activation of inflammatory transcription factors.

One of these oncogenic transcription factors is the calcineurin-responsive Nuclear Factor Of Activated T cells (NFAT) family, originally identified as a group of inducible nuclear proteins, which regulate transcription during T lymphocyte activation [11]. However, a multitude of studies established that NFAT expression and function is not restricted to the immune system, but participates in gene regulation in multiple tissues [12]. The different NFAT isoforms are functional in tumor cells as well as in multiple compartments of the tumor environment and ectopic activation of NFAT family members is now recognized as an important aspect for oncogenic transformation in several human malignancies, most notably in pancreatic cancer [13,14]. In this issue we describe the regulation and activation of NFAT members in pancreatic acinar cells and review the relevance of NFAT-mediated transcription in the sequential steps of pancreatic carcinogenesis.

NFAT Regulation and NFAT-Mediated Transcription in Pancreatic Carcinogenesis

The family of NFAT transcription factors comprises four Calcium/Calcineurin-responsive proteins (NFATc1-c4) [15]. In resting cells, NFAT proteins reside in the cytoplasm in a highly phosphorylated and inactive state [16]. Inflammatory cytokine binding to corresponding receptors causes increased intracellular calcium levels, followed by activation of the phosphatase calcineurin which targets and dephosphorylates NFAT proteins in the cytosol [17]. Subsequently, dephosphorylated NFAT shuttles into the nucleus, where it becomes ubiquitinated for HDM2-dependent proteasomal degradation or protected by GSK3 β -mediated phosphorylation (Figure 1) [18]. Upon stabilization, the transcription factor binds to its consensus sequence GGAAA within target gene promoters and enhancers to promote or repress transcription. NFAT-dependent transcription requires functional and biochemical cooperation with partner proteins that

determine target gene selection and the mode of transcription (repression versus activation) by NFAT proteins [14]. Thus, the nuclear composition and activation status of NFAT-partner complexes highly contributes to the broad spectrum of cellular functions that are attributed to the NFAT family.

NFAT-activities in PDAC and identified additional key functions of the family in the earliest phases of pancreatic cancer initiation, namely the transcriptional induction of acinar-to-ductal metaplasia (ADM).

Implication of NFATc1 in pancreatic cancer initiation

The cellular origin of PDAC has been a controversial topic for many decades. In recent years, increasing evidence indicates that PanIN formation and invasive PDAC originate from differentiated acinar cells via a trans-differentiation program termed acinar-ductal metaplasia. Inflammation-linked activation of the Epidermal Growth Factor Receptor (EGFR) in patients suffering from chronic pancreatitis as well as in early pancreatic cancer stages suggests an involvement of EGFR mediated processes in ADM [19]. However, though accumulating evidence from human and murine studies support an essential role of EGFR signaling activation in inflammation-driven metaplasia and pancreatic cancer initiation [3,20], the molecular mechanisms linking EGFR with ADM remained elusive.

NFAT transcription factors participate in the regulation of genes influencing the development and differentiation of multiple tissues, including chondrocyte differentiation, myogenesis and the development of the cardiovascular system [21,22]. In line with this, recent findings of our group describe a critical involvement of NFATc1 in acinar transdifferentiation. EGFR mediated ADM involves complex formation of NFATc1 and AP1 proteins in the metaplastic epithelial cell and requires NFATc1 dependent transcriptional activation of target genes crucial for ADM formation. Pharmacological or genetic inhibition of NFATc1 in GEMM with a conditional knockout of the transcription factor *KrasG12D;NFATc1^{fl/fl};pdx1-Cre* significantly impaired acinar-to-ductal transdifferentiation. Thus our results link EGFR-NFATc1 signaling with inflammation-induced pancreatic cancer initiation and open new doors for preventive therapeutic options for high-risk pancreatitis patients.

NFAT in proliferation and pancreatic cancer growth

Following their initial discovery, a multitude of studies quickly established that NFAT participates in the regulation of genes influencing differentiation and cell growth. One of the first studies implicating NFAT transcription factors in proliferation and growth was performed in fibroblasts, where constitutive activation of NFATc1 induced colony formation and transformation [23]. In pancreatic tumor cells, proliferation and anchorage-independent growth is - at least in part - dependent on calcineurin activity and nuclear translocation of NFATc1 and NFATc2 [24], underlining the oncogenic impact of both transcription factors in pancreatic cancer. An increased cell-cycle re-entry and the acceleration of G1-S phase transition are appreciated as hallmarks of increased tumor cell proliferation [25,26]. This process is dependent on rapid and sustained induction of immediate early transcription factors (ITF) [27]. Proto-oncogenic c-Myc was identified as a master ITF in G1-S cell-cycle progression in various tumor entities, including PDAC [24]. In untransformed acinar cells, c-Myc activation is repressed by Transforming Growth Factor β (TGF β) - signaling. C-Myc repression by TGF β requires activation of a Smad3/4 repressor complex that binds to the TGF β -inhibitory element (TIE) within the proximal c-Myc promoter, resulting in transcriptional silencing of the gene [28]. However, during pancreatic carcinogenesis, many tumor cells change their transcriptional responsiveness to TGF β and become resistant to its growth-inhibiting

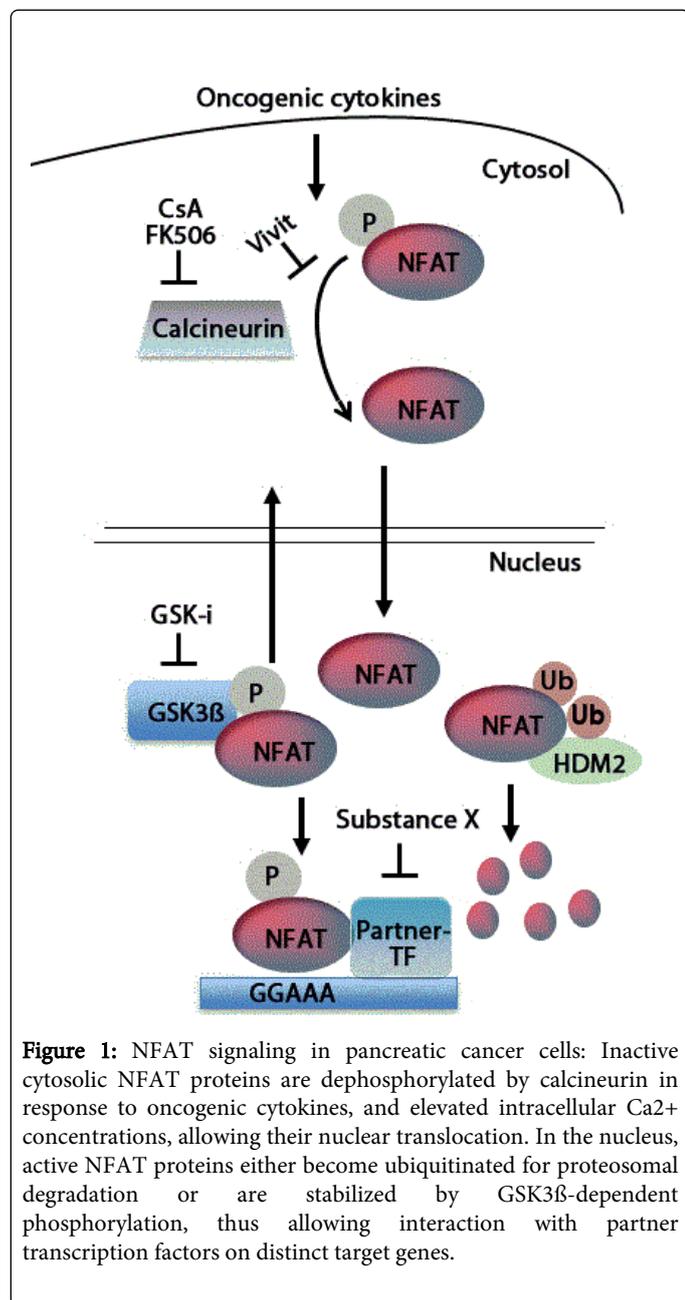


Figure 1: NFAT signaling in pancreatic cancer cells: Inactive cytosolic NFAT proteins are dephosphorylated by calcineurin in response to oncogenic cytokines, and elevated intracellular Ca²⁺ concentrations, allowing their nuclear translocation. In the nucleus, active NFAT proteins either become ubiquitinated for proteosomal degradation or are stabilized by GSK3 β -dependent phosphorylation, thus allowing interaction with partner transcription factors on distinct target genes.

NFAT transcription factors promote pancreatic carcinogenesis via distinct transcriptional programs leading to increased proliferation and growth, inhibition of critical fail-safe mechanisms and interference with key tumor-suppressor functions. In addition, NFAT proteins contribute to tumor cell escape from the adaptive immune system and eventually drive tumor cell dissemination via induction of Epithelial Mesenchymal Transition (EMT) and acquisition of stem cell features, all together characterizing NFAT transcription factors as powerful oncogenes in pancreatic cancer. Recent studies on NFAT-mediated gene regulation further extended the repertoire of oncogenic

effects due to functional or mutational inactivation of the TGF β -Smad pathway [29].

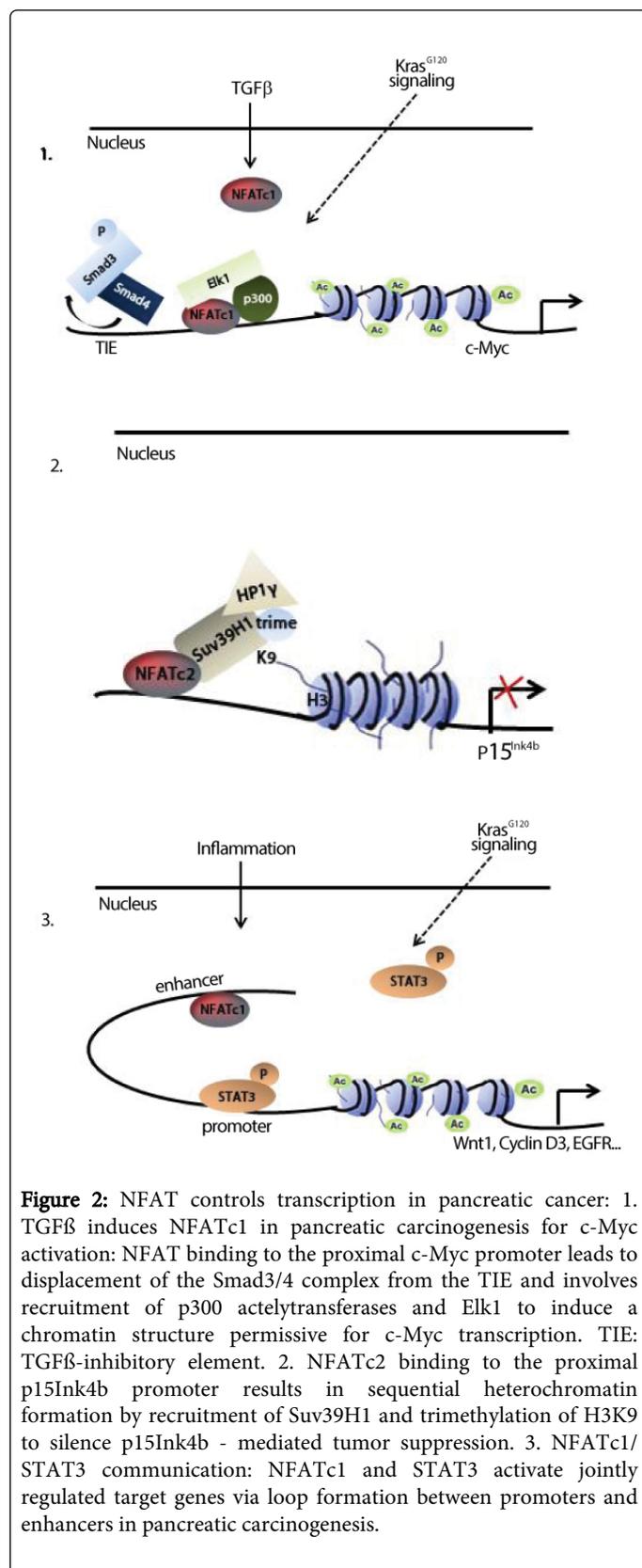


Figure 2: NFAT controls transcription in pancreatic cancer: 1. TGF β induces NFATc1 in pancreatic carcinogenesis for c-Myc activation: NFAT binding to the proximal c-Myc promoter leads to displacement of the Smad3/4 complex from the TIE and involves recruitment of p300 acetyltransferases and Elk1 to induce a chromatin structure permissive for c-Myc transcription. TIE: TGF β -inhibitory element. 2. NFATc2 binding to the proximal p15^{Ink4b} promoter results in sequential heterochromatin formation by recruitment of Suv39H1 and trimethylation of H3K9 to silence p15^{Ink4b} - mediated tumor suppression. 3. NFATc1/STAT3 communication: NFATc1 and STAT3 activate jointly regulated target genes via loop formation between promoters and enhancers in pancreatic carcinogenesis.

In fact, more than 50% of human pancreatic cancers harbour inactivating mutations in the Smad4 (DPC4) tumor suppressor gene [30]. Upon loss of Smad signalling, TGF β can control gene regulation in pancreatic cancer cells through Smad-independent pathways [17], including activation of PI3K and MAPK signaling [29], to activate non-Smad transcription factors. One such factor is NFAT that is highly induced upon TGF β stimulation, and accumulates in the nucleus to govern gene expression. Importantly, NFAT proteins can displace pre-existing Smad3 repressor complexes from the c-Myc TIE element and induce p300-dependent histone acetylation thus rendering the promoter transcriptionally active. Hyperacetylation of the c-Myc promoter is required for the recruitment of the Ets-like gene 1 (ELK-1), a protein that signals downstream of Kras and is crucial for maximal activation of c-Myc (Figure 2) [27]. While NFAT-mediated transcriptional activation of c-Myc increases G1-S phase transition and results in accelerated tumor cell growth *in vivo* and *in vitro*, genetic or pharmacological depletion of NFAT restores TGF β growth suppression in pancreatic cancer cells and impairs c-Myc expression [27,29], thus emphasizing the functional significance of this pathway in pancreatic cancer proliferation.

The oncogenic potential of NFATc1 in pancreatic carcinogenesis and tumor cell proliferation is mirrored in GEMM with constitutive activation of NFATc1 [31]. While mice bearing an acinar cell specific singular constitutive activation of NFATc1 failed to develop advanced PanIN lesions within the observation span of one year, combined pancreas-specific activation of NFATc1 and Kras (Kras^{G12D};NFATc1^{fl/fl};pdx1-Cre), a situation found in 70% of human PDAC diseases, resulted in a dramatic acceleration of pancreatic carcinogenesis and led to an extremely shortened survival compared to Kras^{G12D};pdx1-Cre animals. Further resembling human PDAC, Kras^{G12D};NFATc1^{fl/fl};pdx1-Cre mice developed severe cachexia and abdominal distension due to sanguineous ascites and bile duct obstruction. Upon necropsy, the pancreata of Kras^{G12D};NFATc1 mice displayed enlarged pancreatic tumor masses with both cystic and solid regions and regularly showed liver metastases. This pancreatic cancer GEMM which nicely recapitulates the human disease, hints on synergistic effects of Kras-signaling and NFAT mediated transcription. Recent studies established that NFATc1 partners with the Kras-downstream target signal transducer and activator of transcription-3 (STAT3) [31]. In depth-going investigations including ChIP-Seq combined with genome-wide expression analysis revealed that NFATc1-STAT3 interaction comprises the formation of promoter-enhancer communications on jointly regulated target genes involved in proliferation and growth, including cyclins, Wnt-family members and EGFR (Figure 2).

Summarily, intensive studies in human and murine pancreatic cancer models describe a crucial function of NFAT transcription as a promoter of proliferation and growth in Kras-driven pancreatic carcinogenesis. Moreover, NFAT family members maintain pancreatic tumor cell proliferation and pancreatic cancer progression via inhibition of tumor-suppressive signals.

NFAT Mediated Disruption of Tumor Suppression in Pancreatic Carcinogenesis

Counteracting transformation and growth, cellular senescence, a permanent cell growth arrest, is recognized as one of the most critical fail-safe mechanisms impeding pancreatic carcinogenesis [32]. A major course of this permanent cell-cycle arrest was found in telomeres which shorten progressively during division of normal cells

and force the cell to become senescent, when telomeres erode down below a threshold length [33,34]. Importantly, senescence can also be found in the absence of any detectable telomere shortening, for example as a response to oncogene activation. Oncogene induced senescence (OIS) has emerged as a powerful tumor suppressive mechanism protecting cells from unrestrained proliferation imposed by oncogenic signaling [35]. In response to oncogenic activation of Kras in the pancreas the Ink4 family of cell cycle inhibitors (p15Ink4b, p16Ink4a and p14Arf) is highly induced and blocks cell cycle progression by inhibition of cyclins and their kinases [36,37].

Consistent with their central role in pancreatic tumor suppression, inactivation of p15Ink4b and its close relatives occurs in over 90% of PDACs [26,36]. Additional to mutational or deletion-caused inactivation of the cell-cycle inhibitors, silencing of tumor suppressor genes also occurs via epigenetic events. For example, NFATc2 targets p15Ink4b for inducible and sequential heterochromatin formation. NFATc2 binding to its putative binding site on the proximal p15Ink4b promoter leads to recruitment of the histone methyltransferase Suv39H1. Local trimethylation of lysine 9 on histone 3 (H3K9trime) allows docking of heterochromatin protein 1 γ (HP1 γ) which results in stabilization of the protein complex on the p15Ink4b promoter. Inactivation of NFATc2 disrupts the repressor complex and results in restoration of p15Ink4b expression and tumor-suppressive function (Figure 2) [37].

Additionally to senescence, NFATc2 activation downstream of Wnt5a impedes pancreatic apoptosis and thus promotes drug resistance and tumor cell survival [38]. Moreover, ongoing studies facing pancreatic chemoresistance and metastasis describe antithetical NFATc1 and p53-signaling pathways in pancreatic cancer, suggesting that NFAT transcription might impede p53-induced apoptosis (V.E. personal communication).

Supplementary to senescence and apoptosis tumor suppressive signals extinguish from a physiological immune response that comprises recognition and elimination of transforming cells through CD4- and CD8-positive T-cells [39]. During pancreatic carcinogenesis this cancer-entity independent tumor-suppressive mechanism is impeded by activation of myeloid-derived-suppressor cells (MDSC) in the pancreatic tumor stroma, which insulate pancreatic cancer cells from T-cell mediated inactivation [40]. Preliminary data suggests an essential role of NFAT proteins in the transcriptional induction of a core of cytokines associated with MDSC recruitment and activation (unpublished data). Hence, NFAT does not only counteract tumor suppression in the transforming pancreatic epithelial cell itself, but also contributes to pancreatic cancer progression by conducting the composition of the pancreatic environment.

Perspective

NFAT-dependent oncogenic activity constitutes a frequent event in pancreatic carcinogenesis that occurs in all steps of PDAC development and progression. NFAT-mediated transcription is crucial for PDAC initiation in ADM as well as in PanIN-progression towards invasive adenocarcinoma. Exceeding pancreatic carcinogenesis, NFAT is also functional in maintenance of invasive PDAC and inhabits crucial functions in pancreatic de-differentiation and metastases (unpublished data). The manifoldness of NFAT mediated functions in pancreatic carcinogenesis is - at least in part - due to various partner proteins that combine Kras-dependent signals with NFAT-mediated transcription. As oncogenic activation of Kras, which occurs in nearly

all patients suffering from PDAC, evades any pharmacological intervention, targeting of oncogenic pathways that cooperate with Kras signaling might be a promising strategy for new therapeutic approaches. Three structural unrelated inhibitors, Cyclosporin A (CsA), VIVIT and FK506 display potent Calcineurin/NFAT inhibitors and are widely used as immunosuppressive drugs in tissue transplantation [41]. All three inhibitors prevent nuclear translocation of NFAT by interfering with calcineurin or the calcineurin/NFAT interaction (Figure 1) [11]. As genetic depletion of NFATc1 in mice effectively decelerates pancreatic carcinogenesis [31], one could predict, that the aforementioned inhibitors demonstrate effective cancer therapeutics. Paradoxically, there is a significant increase in cancer incidence in patients on long-term immunosuppressive treatments [42]. This might be caused by the fact that these inhibitors do not specifically target calcineurin/NFAT signaling in the transforming cell, but also inhibit differentiation and activation of tumor cell attacking T-cells. Although our knowledge about NFAT mediated transcription in pancreatic carcinogenesis has been extended within the last years, specific targeting of NFAT in this disease remains challenging. There is a great need for drugs that selectively target tumor-specific NFAT functions, e.g. via modulation of NFAT metabolism or disruption of tumor promoting partner complexes.

Addressing NFAT protein turnover and metabolism, for instance through inhibition of NFATc1 stabilizing kinases (e.g. GSK3 β), might offer interesting new therapeutic strategies *in vitro* and *in vivo* (Figure 1) [18]. Furthermore, accumulating evidence hints on broad NFAT cooperation with chromatin-remodeling proteins as Suv39H1, p300 and EZH2 [37,43-45] that demonstrate promising targets for new therapeutic approaches. Further investigations of NFAT-associated epigenetic mechanisms in pancreatic carcinogenesis will certainly extend the spectrum of pharmacological interference in this disease.

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