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Expression profile and function of Wnt signaling mechanisms in malignant mesothelioma cells

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ABSTRACT

Malignant mesothelioma (MM) is an uncommon and particularly aggressive cancer associated with asbestos exposure, which currently presents an intractable clinical challenge. Wnt signaling has been reported to play a role in the neoplastic properties of mesothelioma cells but has not been investigated in detail in this cancer. We surveyed expression of Wnts, their receptors, and other key molecules in this pathway in well established in vitro mesothelioma models in comparison with primary mesothelial cultures. We also tested the biological response of MM cell lines to exogenous Wnt and secreted regulators, as well as targeting β-catenin. We detected frequent expression of Wnt3 and Wnt5a, as well as Fzd 2, 4 and 6. The mRNA of Wnt4, Fzd3, sFRP4, APC and axin2 were downregulated in MM relative to mesothelial cells while LEF1 was overexpressed in MM. Functionally, we observed that Wnt3a stimulated MM proliferation while sFRP4 was inhibitory. Furthermore, directly targeting β-catenin expression could sensitise MM cells to cytotoxic drugs. These results provide evidence for altered expression of a number of Wnt/Fzd signaling molecules in MM. Modulation of Wnt signaling in MM may prove a means of targeting proliferation and drug resistance in this cancer.

1. Introduction

Malignant mesothelioma (MM) is an aggressive cancer associated with past asbestos exposure that is characterized by rapid progression, late metastases and poor prognosis [1]. This relatively uncommon tumor presents significant clinical challenges since it is highly resistant to conventional therapy, with both radiotherapy and chemotherapy having limited effect [2]. There is an urgent need for more effective therapies for this cancer based upon identification of molecular targets through improved biological understanding of the disease. There is convincing evidence in a number of different cancers that chronic activation of Wnt signaling is important in tumorigenesis and this has fueled interest in targeting this pathway [3].

The Wnts are secreted glycoproteins (at least 19 in humans) that transduce signals by binding to specific frizzled (Fzd) receptor complexes (reviewed in [4]), which activate canonical or non-canonical pathways depending on the make-up of the Wnt/Fzd complex. The best characterised canonical pathway controls β-catenin mediated transcriptional activation of specific gene expression and is thought to have the most significance in cancer development. Loss of control of Wnt signaling pathways has been widely described in cancer and is thought to be an important factor in tumor development [3]. Numerous studies have demonstrated the contribution of Wnts to various aspects of cancer progression including growth and survival signaling, invasion and metastasis, both directly and by crosstalk with other pathways [5].

Aberrations of Wnt signaling have been described in mesothelioma and there is a need to identify the extent to which this pathway drives cell growth and survival in MM. Investigation of the role that Wnt signaling plays in the pathogenesis, progression, and resistance to apoptosis of MM has received limited attention until recently in comparison with other neoplasms. There is evidence for elevated β-catenin protein levels in mesothelioma tumors and models although activating mutations of CTNNB1 (β-catenin) have not been found in MM [6–9]. Several studies have examined specific molecules and reported overexpression of Wnt1, Wnt2, and Dishevelled (Dvl) in mesothelioma cells with

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biochemical consequences for cell proliferation and apoptosis [8,10,11]. Aberrant downregulation of the secreted frizzled-related protein (sFRP) family of Wnt regulators has been reported in MM [12,13] and one member of this family, sFRP4, may suppress growth and induce apoptosis in mesothelioma cells [14]. However these mechanistic studies employed β-catenin deficient MM cell lines [14,15]. Current evidence indicates that MM tissue and cells are overwhelmingly β-catenin positive [7–9,16], suggesting that studies in β-catenin deficient cells may not be particularly relevant to the disease.

In our laboratory we have previously identified differential expression of sFRPs in mouse mesothelioma models [17]. Based on these findings and previous evidence from other authors, we endeavored to more comprehensively characterise expression of Wnt signaling pathway components and targets in human mesothelioma and primary mesothelial cells. We report that mesothelioma cells express a range of Wnt/Fzd mRNA and identify differential expression of specific molecules in mesothelioma cells. In addition we report in mesothelioma cells that canonical Wnt signaling stimulates proliferation and a biological regulator of Wnt signaling inhibits proliferation. Finally, we show that targeting β-catenin in these cells can increase in vitro sensitivity to cytotoxic drugs.

2. Materials and methods

2.1. Cell culture

The malignant mesothelioma cell lines JU77, LO68 and ONE58 (originally derived from pleural effusions of different patients presenting with malignant pleural mesothelioma) were used in this study [18]. Cells were cultured in RPMI 1640 supplemented with 5% fetal bovine serum, 2 mM glutamax, penicillin (100 IU/ml), and streptomycin (100 μg/ml) (all from Life Technologies, Vic., Aust). Recombinant Wnt 3a was obtained from R&D Systems (MN, USA). The mouse 3T3 cell line expressing sFRP4 was a generous gift of Dr. R. Friis (University of Berne, Switzerland). Conditioned media were generated by cultivation of parental 3T3 (control) or 3T3-sFRP4 cells in serum free media for 48 h, the media harvested, clarified by centrifugation (1200 g, 10 min.), and stored at −80 °C until use. Primary mesothelial cells were isolated and cultured as previously described [19]. All cell cultures were grown at 37 °C in a 5% CO_2 humidified atmosphere.

2.2. Reverse transcription PCR (RT-PCR) and real-time RT-PCR

Total RNA was prepared from cell cultures using Ultraspec reagent (Biotecx, TX, USA) and contaminating DNA was removed using RQ1 DNAse (Promega, NSW, Aust.). cDNA synthesis was carried out using a Superscript III first strand synthesis kit (Life Technologies). Gene specific PCR primers were designed using the Primer 3 software (Supplementary Table 1) [20]. Conventional RT-PCR was performed essentially as previously described [21]. Real-time PCR was performed using a standard protocol from the Sensimix SYBR Kit (Bioline, NSW, Aust.) and run on a RotorGene 2000 (Corbett Research, NSW, Australia). Gene expression data normalisation and analysis were performed as previously described [21].

2.3. RNAi

RNA-i mediated gene silencing of β-catenin was performed using an optimal siRNA duplex previously determined in our laboratory (unpublished data). Transfection of siRNA duplexes was performed at an empirically determined optimal concentration using Lipofectamine (Life Technologies), essentially according to the manufacturer's recommendation. The siRNA duplexes targeting β-catenin (β-cat-608, 5′-AACATGCAGTTGTAAACTTGATT-3′) and control (5′-CGAATCCTACAAAGCGCGC-3′) were purchased from Proligo (N.S.W., Australia). The control siRNA was derived from *Euglena gracilis* chloroplast ribosomal RNA with no homology to human sequences. Relative expression levels of the interferon-induced 2′–5′ human oligoadenylate synthetase 1 (hOAS1) mRNA were used as a γ-Interferon response control in gene silencing experiments.

2.4. Cell viability

An MTT assay was used to quantitate cell death/viability in transfected cells following 48 h exposure to cisplatin or gemcitabine. Cells were seeded into 96-well plates at a density of 10,000 cells/well. Following 24 h incubation, cells were transfected with siRNA duplexes as above and, following a further 24 h, drugs were added at concentrations from 0–100 μM. After 48 h the MTT assay was performed as previously described [22]. IC_{50} was defined as the concentration causing a 50% reduction in absorbance relative to the negative control. IC_{50} was determined by non-linear regression analysis using Graphpad Prism v4 (Graphpad Software, CA, USA).

2.5. Proliferation

Cell were seeded at a density of 5×10^4 cells/well in 24 well plates and treatments added at 24 h. The cells were harvested 48 h later by trypsinization and evaluated by a trypan blue assay using a Countess Automated Cell Counter (Life Technologies).

2.6. Statistical analysis

Statistical comparison between two groups was performed using unpaired *t*-test with Instat (Graphpad Software). The difference was determined to be statistically significant if $p < 0.05$.

3. Results

3.1. Expression of Wnt, frizzled and other signaling molecules in mesothelioma cells

RT-PCR analysis was used to determine which Wnt-related genes were expressed by three mesothelioma cell lines as well as three primary mesothelial cell cultures. cDNAs from mesothelial cells obtained from three different donors were analysed, and expression patterns were found to be generally similar across all cell populations. Wnt 2b, 3, 4, 5a, and 10b expression was identified in all cells, whereas Wnt 6 was clearly detected only in ONE58 cells (Fig. 1A). Expression of Wnt 1, 2, 3a, 7a, 8a, 10a, and 16 transcripts were not detected in either tumor or primary cells using this method (data not shown). The expression of all 10 frizzled receptors was also assayed and expression of these showed more variability across the cells. Fzd 4, 6, and to a lesser extent Fzd 2 and 3, were consistently expressed while Fzd 1, 5, 7, 8, and 10 were detected in some cells (Fig. 1B) but Fzd 9 was not detected (data not shown).

Other signaling molecules such as LRP-5, LRP-6, APC, and β-catenin as well as the downstream Wnt targets c-Myc, axin2 and cyclin D1 were also shown to be expressed by these cells (Fig. 2A). Notably, the transcription factor LEF1 was expressed in mesothelioma cells but was not detected in mesothelial cultures.

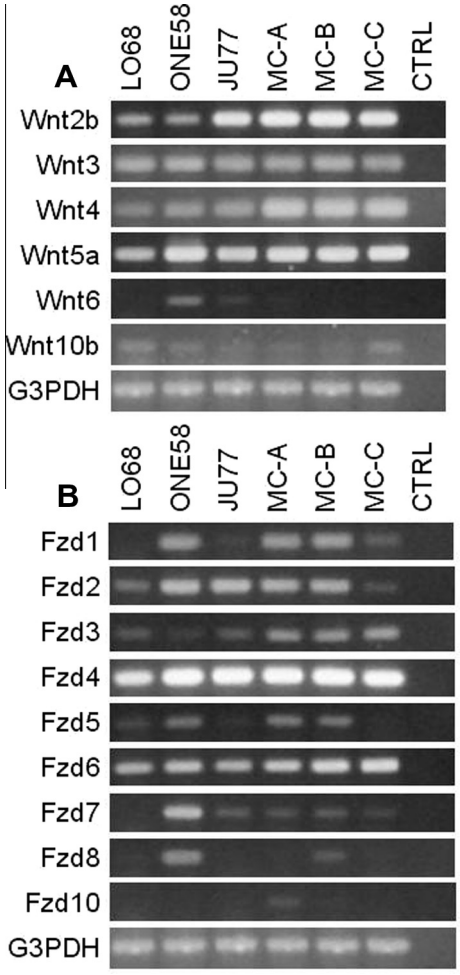

Fig. 1. (A) Wnt ligand and (B) Frizzled receptor mRNA expression in mesothelioma cell lines and mesothelial cell cultures. Total RNA isolated from cells was analysed by 2-step RT-PCR using gene specific primers. Wnts 1, 2, 3a, 8a, 7a, 10a, 16 and Fzd 9 were not detected.

3.2. sFRPs are differentially expressed in mesothelioma and mesothelial cells

Since there is evidence for aberrant expression of secreted regulators of Wnt signaling in mesothelioma [12], the expression of all 5 sFRPs was examined by RT-PCR in these cells. The sFRP 1, 2, 3, and 4 transcripts were detectable in some cultures (Fig. 2B) whereas sFRP 5 was not found under these conditions (data not shown). Strikingly, sFRP4 mRNA was expressed in all primary mesothelial cell cultures but was not detected in any of the mesothelioma tumor cell lines.

3.3. Relative Wnt- related gene expression in mesothelioma and mesothelial cells

The RT-PCR data (Figs. 1 and 2) was suggestive that for some genes there was differential expression between mesothelial and mesothelioma cells. Expression of these genes was further investigated using real-time RT-PCR to quantitate relative levels of these transcripts. These assays revealed large differences in expression levels of some Wnt pathway genes (Fig. 3). Wnt2b was downregulated in LO68 and ONE58 cells relative to mesothelial cells although this was not as pronounced in JU77 cells (Fig. 3A). Notably, Wnt4 mRNA expression was over a 1000-fold lower in the tumor cell lines relative to primary mesothelial cells (Fig. 3B). Both the negative targets of Wnt signaling (axin2 and APC) were over

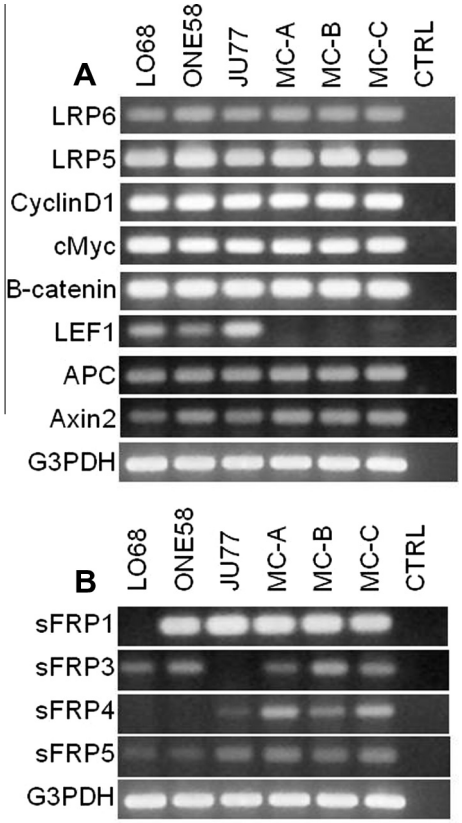

Fig. 2. Expression of mRNA of (A) sFRP and (B) Wnt pathway components and targets in mesothelioma cell lines and mesothelial cell cultures. Total RNA isolated from cells was analysed by 2-step RT-PCR using gene specific primers.

a 100-fold downregulated in mesothelioma cell lines (Fig. 3C and D). In contrast, the positive targets of Wnt signaling, c-Myc and cyclin D1, were not differentially expressed, with the exception of c-Myc in LO68 (Fig. 3E and F).

3.4. Wnt signaling molecules modulate mesothelioma cell proliferation

Having examined expression of Wnt signaling pathway molecules and targets, we next investigated the functional effects of this pathway in these cells. Initially we examined the effect of the secreted regulator of Wnt signaling, sFRP4, which we had found to be downregulated in mesothelioma cells (Fig. 2B). Previous studies have shown growth repression in β-catenin null mesothelioma cells [14]. However, all of our mesothelioma cell lines express β-catenin (Fig. 2A) and we were interested to see the effect of this molecule in mesothelioma cells that had functional canonical signaling. We found that sFRP4 conditioned media suppressed cell proliferation/viability in JU77 and LO68 cells but not ONE58 (Fig. 4A). We next determined the effect of the canonical Wnt ligand, Wnt3a, upon proliferation in these cells. The effect of exogenous Wnt3a was to stimulate proliferation of mesothelioma cells with significant upregulation seen in JU77 and to a lesser extent in ONE58 (Fig. 4B). These results indicate that Wnt signaling molecules can modulate proliferation in mesothelioma cells.

3.5. β-Catenin signaling mediates mesothelioma cell drug sensitivity

Given the effects of Wnt3a and sFRP4 on these cells, we examined whether targeting canonical signaling might influence cytotoxic drug sensitivity. We used RNAi mediated knockdown to target β-catenin (Fig. 4E) and then determined the sensitivity of JU77 and ONE58 to cisplatin and gemcitabine (Fig. 4C and D). In

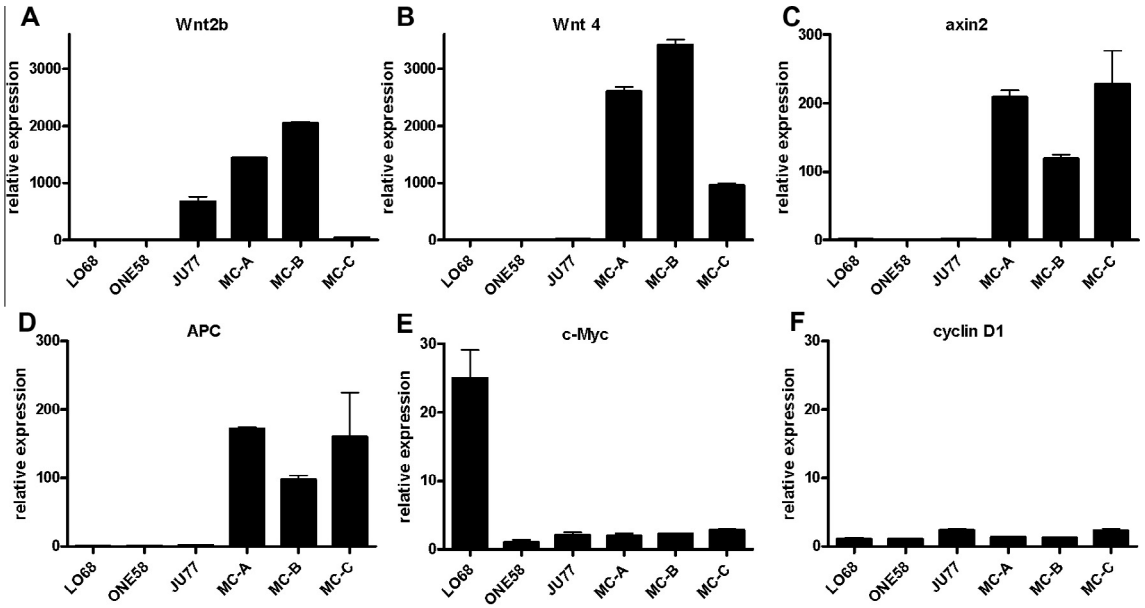

Fig. 3. Basal gene expression of (A) Wnt2b, (B) Wnt4, (C) axin2, (D) APC, (E) c-Myc, and (F) cyclin D1 in mesothelioma and primary mesothelial cells (MC). Total RNA isolated from cells was analysed by 2-step real-time RT-PCR using gene specific primers. Basal gene expression is expressed as mRNA levels relative to the lowest expressing culture following normalisation by reference gene expression. Results are mean ± SD for three cultures.

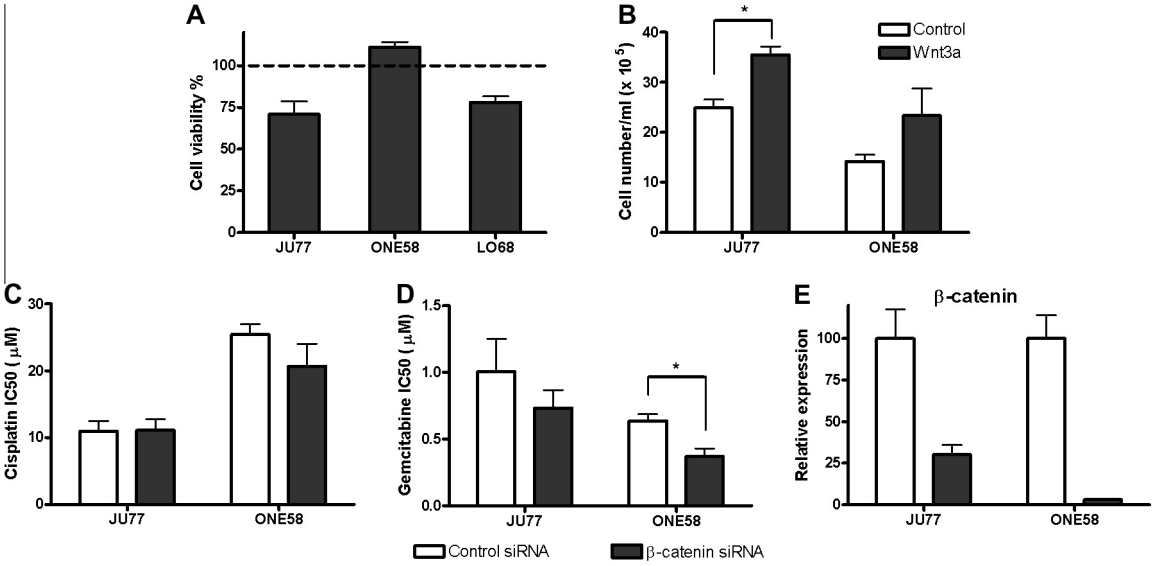

Fig. 4. Wnt signaling molecules modulate mesothelioma cell proliferation and survival. (A) Cells were cultured in sFRP4 conditioned media for 72 h and viability assayed by MTT. Results are percent relative to control conditioned media. (B) Cultures were treated with 250 ng/ml recombinant Wnt3a for 48 h and proliferation determined by trypan blue assay. Following RNAi mediated β-catenin knockdown, cells were treated with (C) cisplatin (0–100 μM) or (D) gemcitabine (0–100 μM) for 48 h and IC_{50} determined by non-linear regression analysis of MTT data. (E) Cells were transfected with β-catenin or control siRNA and β-catenin mRNA levels assayed at 48 h by real-time PCR. All results are mean ± SEM of at least 3 independent experiments. $^{*}p < 0.05$.

ONE58 cells, β-catenin knockdown was most effective and significantly enhanced the cytotoxicity of gemcitabine (Fig. 4D).

4. Discussion

Previous studies of Wnt signaling molecules in mesothelioma have mainly investigated the role of selected molecules. In the present study, we examined expression of all Wnt ligands and Frizzled receptors as well as a number of other key molecules. We found 5 Wnts and 3 FZDs commonly expressed in our cells. Significantly, LEF1 was expressed in mesothelioma cells but not in mesothelial cells. LEF1 binds to Wnt responsive elements in DNA and, principally through interaction with β-catenin, activates transcription of numerous genes depending upon molecular context. Aberrant overexpression of LEF1 has been identified in a number of cancers and has been associated with poor prognosis in chronic lymphocytic leukemia [23], prostate cancer [24] and colorectal cancer [25]. The functional effects of LEF1 in tumors include enhancement of growth and invasiveness [24,26]. Furthermore, both in vitro and in vivo experiments have indicated that overexpression of LEF1 can result in neoplastic transformation [27,28]. LEF1 overexpression in mesothelioma cells is consistent with aberrant activation of this pathway.

Wnt4 is generally classified as a non-canonical Wnt, however, there is evidence that in some contexts Wnt4 can signal through

β-catenin [29]. Although Wnt4 has been shown to have an important developmental role including haematopoesis [30], kidney, and sex development [31,32], there is only limited evidence regarding the molecule in cancer. Wnt4 expression is induced by the tumor suppressors p63 and p73 [33] and can antagonise canonical signaling by Wnt3a [34] or, in cancer cells, inhibit Ras induced cell migration [35]. The downregulation of Wnt4 we report here is intriguing in this context because few studies have reported downregulation of Wnts in cancer. Further investigation is required to reveal the consequences of this finding in mesothelioma cells.

Both AXIN2 and APC are involved in negative regulation of Wnt signaling and were found to be downregulated in the present study. They are often described as target genes for canonical signaling as part of a regulatory feedback mechanism [36]. However, a number of studies have demonstrated epigenetic downregulation of these genes in some cancers [37–39] and, more recently, positive regulation of both AXIN2 and APC by CDX2 tumor suppressor [40]. Further investigation is required as to whether the mechanism underlying activation of Wnt signaling in mesothelioma cells is sensitive to variation in expression of these genes as has been reported in colon cancer cells [37].

With a significant role in the determination of cell fate, Wnt signaling is regulated via a diverse array of strategies including secretory factors among which are the sFRPs, which are structurally related to the FZD receptors [41]. Previously it has been reported that expression of sFRPs 1, 3, 4, and 5 were apparently downregulated in mesothelioma tissue samples and two cell lines [12], and that sFRP4 may suppress growth and induce apoptosis in β-catenin null mesothelioma cells [12,14]. Our findings of sFRP4 downregulation are consistent with these reports and also our own similar results in mouse mesothelioma models [17]. That we found both sFRP4 and Wnt3a may modulate mesothelioma cell growth in more biologically relevant models is also significant since most mesotheliomas express β-catenin [7,16].

Overall, our results demonstrate expression of key components of Wnt signaling in mesothelioma cells and the differential expression of a number of molecules, which have not previously been reported. Furthermore, exogenous Wnt can stimulate mesothelioma cell proliferation while secreted regulators can inhibit it. Notably, we found that targeting Wnt signaling can render mesothelioma cells more sensitive to cytotoxic drugs. Further investigation is required to examine whether the development of therapeutic strategies targeting this pathway may lead to novel treatments for this intractable cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.025.

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