Review

Advance Publication DOI: 10.5582/bst.2017.01043

The T-Box transcription factor 3 in development and cancer

Tarryn Willmer, Aretha Cooper, Jade Peres, Rehana Omar, Sharon Prince*

Department of Human Biology, Faculty of Health Sciences, Anzio Road, University of Cape Town, Cape Town, South Africa.

Summary **T-box factors comprise an archaic family of evolutionary conserved transcription factors that regulate patterns of gene expression essential for embryonic development. The T-box transcription factor 3 (TBX3), a member of this family, is expressed in several tissues and plays critical roles in, among other structures, the heart, mammary gland and limbs and haploinsufficiency of the human *TBX3* gene is the genetic basis for the autosomal dominant disorder, ulnar-mammary syndrome. Overexpression of *TBX3* on the other hand has been linked to several cancers including melanoma, breast, pancreatic, liver, lung, head and neck, ovarian, bladder carcinomas and a number of sarcoma subtypes. Furthermore, there is strong evidence that TBX3 promotes oncogenesis by impacting proliferation, tumour formation, metastasis as well as cell survival and drug resistance. More recently, TBX3 was however shown to also have tumour suppressor activity in fibrosarcomas and thus its functions in oncogenesis appear to be context dependent. Identification of the upstream regulators of TBX3 and the molecular mechanism(s) underpinning its oncogenic roles will make valuable contributions to cancer biology.**

Keywords: TBX3, transcription factor, embryogenesis, oncogenesis

1. Introduction

The T-box family of transcription factors are highly conserved through evolution and based on phylogenetic analysis, the T-box gene family is divided into five subfamilies, namely *Brachyury* (*T*), *T-brain* (*Tbr1*), *TBX1*, *TBX2*, and *TBX6* (*1*). Members of this family are defined by a unique DNA binding domain known as the T-box which *in vitro*, binds a partially palindromic sequence T(G/C)ACACCT AGGTGTGAAATT, known as the T-element, as well as single T-element half sites in multiple orientations (*2*). *In vivo* data have, however, revealed that the T-element binding site is highly variable and degenerate and there is evidence that T-box factors can also bind their target genes through co-factor binding sites (*3,4*). Members of the T-box family have well-established roles in the development of vertebrate and invertebrate species where they are expressed in a wide array of tissues and their functions range from early cell-fate decisions to organogenesis (*5*). Their critical roles in development are evident by the number of human congenital developmental syndromes associated with mutations in *T-box* genes (*6*). In addition, there is overwhelming evidence implicating T-box factors in cancer biology where they behave as oncogenes and/or tumour suppressors (*7*).

2. TBX3

The T-box transcription factor, TBX3, is critical for development and has emerged as an important player in the oncogenic process. It is essential for the formation of, amongst other structures, the heart, limbs, mammary glands, teeth and genitalia and haploinsufficiency of the human *TBX3* gene results in ulnar-mammary syndrome (UMS) which is characterised by malformations of these organs and body structures. On the other hand, the overexpression of *TBX3* is a feature of a wide range of cancers and it has been implicated in several aspects of the oncogenic process ranging from the bypass of senescence to the inhibition of apoptosis, migration and invasion. However, little is known about the molecular basis for the role of TBX3 in development and oncogenesis because there is a paucity of information

Released online in J-STAGE as advance publication June 4, 2017.

**Address correspondence to:*
Dr. Sharon Prince, Department of Human Biology, Faculty of Health Sciences, University of Cape Town, Observatory, 7925, Cape Town, South Africa.
E-mail: sharon.prince@uct.ac.za

regarding its upstream regulators, its target genes as well as the protein co-factors that it co-operates with to execute its functions.

2.1. *TBX3 gene and protein structure*

Tbx3 forms part of the *Tbx2* subfamily of T-box factors, which comprise the closely related *Tbx2*, *Tbx3*, *Tbx4*, and *Tbx5*, all of which originated from a single ancestral gene (*8*). Based on the branch length of their phylogenetic tree, it is proposed that *Tbx3* was duplicated by unequal crossing over events, giving rise to two gene clusters, namely *Tbx2/Tbx3* and *Tbx4/Tbx5*. An additional duplication of this cluster gave rise to four separate genes, with *Tbx2* and *Tbx4* being situated on chromosome *17q23* and *Tbx3* and *Tbx5* on chromosome *12q24* in humans (*8-10*). Due to the original duplication event, TBX3 is more closely related to TBX2 and in humans they share a 95% identity within their DNA binding domain and approximately 70% homology in their N-termini (*11*).

In humans, *TBX3* maps to the reverse strand of chromosome *12* at position *12q23-24.1* and alternative splicing results in a number of transcript variants with *TBX3* and *TBX3+2a* being the most extensively studied (*9*). Fan *et al*. showed that, while *TBX3* and T*BX3+2a* are widely expressed in both mouse and human tissue, their expression ratio is species and tissue dependent (*12*). The human *TBX3* coding region spans approximately 4.7 kb, contains 7 exons and encodes a 723 amino acid protein (Figure 1). Differential splicing of the second intron, however, results in the addition of the 2a exon, producing the TBX3+2a isoform with an additional 20 amino acids in the T-box domain (*12,13*). It is still unclear whether these variants have overlapping or distinct roles and whether the +2a insertion results in TBX3+2a regulating different target genes to TBX3. Indeed, Fan *et al*. reported that the isoforms were functionally distinct as, while the overexpression of TBX3 inhibited senescence in mouse embryonic fibroblasts (MEFs), the overexpression of TBX3+2a promoted this process (*12*). These functional differences were ascribed to the fact that TBX3, and not TBX3+2a, was able to bind to the consensus T-element *in vitro*. These findings were contradicted by Hoogaars *et al*. who showed that both isoforms bound the consensus T-element in *in vitro* binding assays, repressed the T-elements previously identified in the *Natriuretic peptide A* (*Nppa*) and *p21WAF1* promoters, and interacted with the homeobox co-factor NK2 homeobox 5 (Nkx2.5) (*14*). In addition, using an *in vivo* transgenic mouse model they showed that TBX3 and TBX3+2a were capable of inhibiting heart chamber formation through repression of the cardiac chamber markers *Nppa* and *Gap junction alpha-5* (*Gja5*). Furthermore, a more recent study suggested that, while the overexpression of Tbx3 and Tbx3+2a inhibited *Nanog* promoter activity in pluripotent mouse embryonic

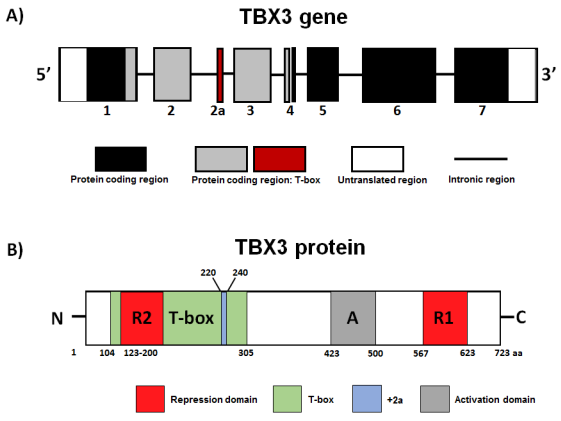

Figure 1. TBX3 gene and protein. Schematic diagram of **(A)** *TBX3* gene including protein-coding sequences (filled boxes), introns (lines), untranslated sequences (white boxes), highly conserved T-box sequences (grey and red boxes) and exon 2a (red box) which is alternatively transcribed; and **(B)** TBX2 protein showing repression domains R1 and R2 (red boxes), T-box (green boxes), activation domain (grey box), additional 20 amino acids in TBX3+2a protein splice form (blue box). The number of amino acid residues is shown under each box (adapted from Bamshad *et al*. 1999).

stem cells (mESCs), only Tbx3+2a physically interacted with Nanog (*15*). Lastly, Kumar *et al*. highlighted a novel role for TBX3 in the regulation of RNA splicing and showed that both isoforms regulated this process (*16*). In summary, while more work is needed to clarify these conflicting findings, it seems likely that the expression pattern and roles of TBX3 and TBX3+2a may vary depending on the cellular context.

The functional domains of the TBX3 protein (Figure 1B) were mapped using a Gal4 binding domain fused to different regions of Tbx3 on a luciferase reporter driven by four proximal promoter Gal4 binding sites (*17*). Constructs expressing either the full length TBX3 protein or the 123-200 or 567-623 regions of the protein led to a repression of relative luciferase activity. This indicated that TBX3 was a transcriptional repressor and identified regions 567-623 and 123-200 as repression domains, which the authors called R1 and R2, respectively. Indeed, TBX3 was subsequently shown to transcriptionally repress *p14ARF/p19ARF*, *p21WAF1*, *Nppa*, *E-cadherin*, and *phosphatase and tensin homolog* (*PTEN*) (*7*). Furthermore, when R1 or R2 were fused to the VP16 (herpes simplex virus protein 16) activation domain and tested in similar experiments, R1 but not R2, was able to override the VP16 activation domain and to repress transcriptional activity (*17*). This led to the suggestion that R1 is the dominant repression domain. A putative activation domain at amino acids 423-500 was also mapped and indeed TBX3 has been shown to activate *Connexin43* (*18*) and *Gata6* (*19*) which are both important in heart embryogenesis. Using electrophoretic mobility shift assays (EMSA) the DNA-binding region was found to be located in the N-terminus (position 105-287 (REFSEQ: accession NM 005996.3)) and a

nuclear localization signal (NLS) was found at residues 292 to 297 (*17*). Bamshad *et al*. characterised mutations in 75 UMS sufferers, from 8 different families and reported that 50% of the mutations resided in the exons encoding the T-box domain and the other 50% occurred in downstream exons which encode the C-terminus (*13*). Together, these reports highlight the importance of the T-box DNA binding domain and C-terminal regions of TBX3. Further characterisation of the structure of the TBX3 protein and its interaction with DNA were shown by Coll *et al*. when they solved the crystal structure of the TBX3 DNA-binding domain (*20*). While they demonstrate that TBX3 binds the palindromic consensus T-box binding site as two independent monomers, it is predicted that TBX3 will bind its physiological targets as a single monomer.

As is the case with other T-box factors, it is speculated that post-translational modifications and interaction with co-factors may regulate TBX3 target gene specificity within different cellular contexts but these areas remain poorly understood (*21*). Indeed, in addition to repressing its known target genes through direct binding to a T-element it can also recruit histone deacetylase (HDAC) 1, 2, 3 and 5 to epigenetically silence promoters and the details will be discussed under relevant sections of this review. Interestingly, mass spectrometry analysis performed in the Moon laboratory revealed that TBX3 interacts with a number of RNA-binding and -splicing proteins which indicated novel roles for TBX3 (*16,22*). Furthermore, they showed that mutations in the TBX3 NLS, representative of those seen in UMS, disrupt its interaction with its RNA-binding partners. They also reported that TBX3 binds its target RNAs through T-elements present within mRNA transcripts and could directly regulate the alternative splicing of *Disks large homolog* (*3Dlg3*) and *Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1* (*Nfκb1*). TBX3 was also shown to facilitate the recruitment and docking of other RNA-binding proteins and splicing factors, such as the RNA helicase, DEAD box helicase 3 (DDX3), to T-element containing RNAs. Interestingly, the UMS mutations present within the C-terminal region of TBX3 interfered with its RNA splicing functions.

The above findings demonstrate that TBX3 can regulate gene expression at multiple levels.

2.2. *TBX3 in development and in stem cell biology*

During mouse development *Tbx3* is first expressed in the morula, then in the inner cell mass of the blastocyst and at the onset of gastrulation it localises to the proximal pole of the epiblast (*23*). It is subsequently found in distinct areas of the developing nervous system, musculo-skeleton, eye, heart, kidney, lung, liver, pancreas and mammary gland (*24*). *TBX3* is also widely expressed in a number of human foetal organs and tissues, including heart, lung, kidney, liver and spleen (*13*). Consistent with its extensive expression pattern, TBX3 contributes to the formation of many tissues with its most well characterised roles being in heart, mammary gland and limb development (*24*). As mentioned earlier, haploinsufficiency of *TBX3* causes the human autosomal dominant UMS which is characterised by a wide range of congenital abnormalities including malformations of the limbs, mammary gland aplasia, loss of areola and defects in the jaw, heart and genitalia (*24*). These abnormalities can also be observed in mouse models of UMS. For example, homozygous mice die *in utero* around embryonic day E12.5 due to heart abnormalities and embryos exhibit mammary gland aplasia and posterior limb abnormalities (*25*). Heterozygous mice exhibit minor aberrations of the external genitalia and have a significantly higher incidence of failed nipple and ductal tree development at E18.5 and adult virgins have aplasia of the first three pairs of mammary glands (*25,26*). It is important to note that there is a large number of tissues and organs where TBX3 is expressed but which are unaffected by UMS, suggesting that specific doses of TBX3 may be required for its functions in different tissues. This is in line with observations that the severity of the UMS phenotype correlates with the level of *TBX3* deficiency (*24*). It is also possible that other T-box factors, such as TBX2, could compensate for TBX3 in tissues and organs unaffected by UMS.

Consistent with the very early expression of *Tbx3* in the inner cell mass of the blastocyst are numerous studies showing that TBX3/Tbx3 can promote both self-renewal and differentiation of embryonic stem cells (ESCs) which play important roles in maintaining homeostasis during embryonic development (*27*). The maintenance of ESC pluripotency involves orchestrated signalling pathways and transcriptional networks including the leukaemia inhibitory factor/signal transducer and activator of transcription (LIF/STAT) pathway, as well as the pluripotency and self-renewal transcription factors, octamer-binding transcription factor 4 (Oct4), Nanog and Sox2. Ivanova *et al*. showed that while *Tbx3* levels were high in undifferentiated mouse ESC (mESC), they were significantly down-regulated in mESCs undergoing retinoic acid (RA) induced differentiation (*28*). They also demonstrated that high levels of *Tbx3* could block differentiation into mesoderm, ectoderm, trophectoderm and neural crest cells. In support of these findings, Tbx3 was shown to be sufficient to maintain self-renewal of mESCs downstream of LIF/STAT signalling (*29*). Interestingly, microRNA (miRNA)-137, which plays important roles in neural stem cell differentiation, was recently shown to directly repress *Tbx3* levels by binding its 3' UTR in mESC (*30*). This repression was also shown to result in disruption of mESC self-renewal and accelerated differentiation *in vitro*. More recently, Cioffi *et al*. also showed that miR-93 represses *Tbx3* and thereby counteracts self-renewal in early adipocyte precursors (*31*).

Lu *et al*. also showed that Tbx3 overexpression in mESCs promotes differentiation into the extra-embryonic endoderm (ExEn) lineage through direct regulation of the ExEn regulator, *Gata6* (*19*). Furthermore, they showed that Tbx3 knockdown during embryoid body formation prevented extra-embryonic endoderm differentiation but enhanced ectoderm and trophectoderm differentiation. In addition, Zhao *et al*. showed that the overexpression of either Tbx3 or Tbx3+2a in ESCs or induced pluripotent stem cells (iPSCs) could induce differentiation by inhibiting the transcriptional activity of Nanog (*15*). This was further demonstrated by studies showing that Tbx3 is highly expressed in definitive endoderm progenitor cells and as mentioned earlier, complexes with the histone demethylase, JmjC domain-containing protein 3 (Jmjd3), to transcriptionally activate *Eomes* and promote endoderm differentiation (*32,33*). Another mechanism by which Tbx3 promotes endoderm differentiation is through the canonical Wingless (Wnt) signalling pathway, where Tbx3 is a downstream effector of Wnt3a and promotes the induction of a primitive endoderm state, giving rise to visceral endoderm (*34*). Interestingly, Waghray *et al*. recently showed using expression studies and chromatin immunoprecipitation (ChIP) sequencing that Tbx3 directly represses *Developmental pluripotency-associated protein 3* (*Dppa3*) at a region 1.7 kb upstream of its transcriptional start site and consequently induces differentiation of ESCs towards the mesoderm (*35*). In addition to this, they identified a Wnt/Tbx3/Dppa3 signalling axis that regulates the balance between mESC self-renewal and differentiation, which may provide one possible explanation for how Tbx3 may either promote or inhibit differentiation.

Tbx3 has also been implicated in the generation of mouse iPSCs by, in part, directly activating the *Oct4* promoter (*36*). In addition, Tbx3 was shown to improve the generation of porcine iPSCs when co-expressed with Oct4, Sox2, Kruppel-like factor 4 (Klf4) and c-Myc (*36*). Furthermore, iPSC formation increased when *Tbx3* expression was enhanced by Zinc finger protein of the cerebellum 3 (Zic3) (*37*). Ectopic overexpression of Tbx3 in the atria of the adult mouse heart was also shown to reprogramme mature differentiated atrial myocytes into a more naïve pacemaker phenotype by repressing genes required for the working myocardial function and stimulating genes required for pacemaker functions (*38*). ESCs and iPSC cells can sporadically enter a "two-cell-like embryonic state" (2C-state) which is important for ESC potency (*39*). Dan et al. showed that in 2C mESCs, Tbx3 could activate the promoter activity of a 2-cell gene, *zinc finger and SCAN domain-containing protein 4* (*Zscan4*) and that ectopic overexpression of Tbx3 in these cells resulted in telomere lengthening and genomic stability (*40*).

The role of TBX3 in promoting differentiation of human ESC (hESC) is somewhat different to that of mESCs, as it promotes neuroepithelial differentiation but not endoderm differentiation (*41*). This is possibly due to the different pluripotent states of hESC and mESCs, with mESCs being in a more naïve state (*42*). TBX3 overexpression cell culture models showed that it could promote hESC proliferation by repressing *p14ARF* and *NFκBIB*, an inhibitor of the NF-kappaB (NF-κB) pathway (*41*). The knockdown of TBX3 on the other hand resulted in decreased expression of neuroepithelial and neuroectoderm markers paired box 6 (PAX6), LIM homeobox 2 (LHX2), forkhead box G1 (FOXG1), and retina and anterior neural fold homeobox (RAX). Taken together, these studies reveal important, context-dependent roles for TBX3 in the maintenance, self-renewal and differentiation of ESC populations.

2.3. *TBX3 and cancer*

TBX3 is overexpressed in a range of carcinomas (breast, pancreatic, melanoma, liver, gastric, lung, head and neck, ovarian and bladder) and sarcomas (chondrosarcoma, fibrosarcoma, liposarcoma, rhabdomyosarcoma and synovial sarcoma) (*7,43,44*). Importantly, there is substantial evidence that this overexpression contributes to the oncogenic process at multiple levels including the bypass of senescence and apoptosis as well as the promotion of proliferation, tumour formation and invasion (Figure 2). In addition, recent studies suggest that there are cancer contexts where TBX3 may also function as a tumour suppressor (Figure 2). Given the myriad of cancer processes that TBX3 impacts it is expected to co-operate with other oncogenic factors and to regulate several target genes. There is, however, limited information regarding these areas and nothing is known about what enables TBX3 to switch between tumour promoter and tumour suppressor.

2.3.1. *TBX3 in senescence, apoptosis and proliferation*

In response to endogenous or exogenous stress signals such as oncogenic stimuli, cells can undergo cell cycle arrests, senescence or apoptosis which protect against inappropriate cell division and/or survival. These processes serve as important barriers to cancer and are regulated at a molecular level by the p14ARF/p53/p21 and p16INK4a/retinoblastoma protein (pRB) tumour suppressor pathways. The inhibition or bypass of these pathways are required for cancer initiation and progression as well as the development of anti-cancer drug resistance.

Senescence is an irreversible/permanent exit from the cell cycle and Tbx3 can bypass this process and consequently immortalise mouse embryonic fibroblasts (MEFs) and ST.HdhQ111 striatal cells through either directly binding or repressing a T- element in the initiator of *p19ARF/p14ARF* or by recruiting HDAC 1, 2, 3 and 5 to epigenetically silence its promoter (*7,45*). A recent study by Kumar *et al*. demonstrated that TBX3 can also bypass senescence by indirectly repressing

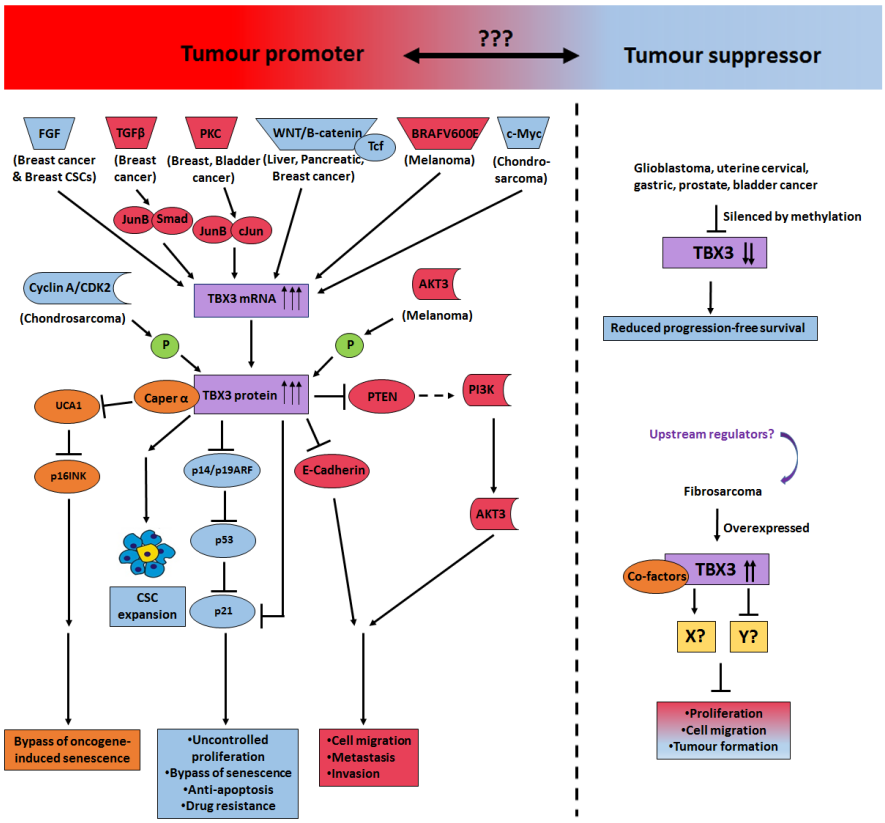

Figure 2: Mechanisms by which TBX3 contributes to cancer. Left panel: TBX3 is overexpressed in numerous cancers where it promotes several aspects of the oncogenic process including the bypass of senescence, uncontrolled proliferation, migration, metastasis and drug resistance. In some cancers, the key oncogenic signalling pathways responsible for this overexpression have been identified (see cancer types in brackets and colour coding which match the appropriate oncogenic processes). **Right panel:** TBX3 also exhibits tumour suppressor activity. It is silenced by methylation in certain cancers and it retards proliferation, cell migration and tumour formation of fibrosarcomas. The factors upstream of TBX3 as well as the co-factors and target genes that mediate the tumour suppressor functions of TBX3 are yet to be elucidated.

p16INK4a (*22*). Indeed, the authors show that Tbx3 in complex with Coactivator of AP1 and Estrogen Receptor (CAPERα) represses the long non-coding RNA, *Urothelial Cancer Associated 1* (*UCA1*) and this prevents the stabilization of *p16INK4a* mRNA (*22*). Furthermore, they showed that RAS-induced senescence of human foreskin fibroblasts resulted in dissociation of the CAPERα/TBX3 complex and a concomitant increase in *UCA1* and *p16INK4a* expression. It would be worth investigating whether the CAPERα:TBX3 interaction and *UCA1* repression is required for senescence bypass in TBX3 driven cancers.

Apoptosis, a form of programmed cell death, plays an essential role in the maintenance of tissue homeostasis and negative regulation of this cellular process promotes malignant transformation and chemoresistance (*46*). There is evidence that TBX3 may contribute to oncogenesis and confer anti-cancer drug resistance by compromising the apoptotic pathway. Indeed, TBX3 co-operated with the Myc oncogene to transform MEFs, where it promoted the bypass of Myc induced apoptosis through the downregulation of p19ARF and p53 levels (*47*). In addition, TBX3 has been shown to inhibit apoptosis in rat bladder hyperplastic epithelial cells and TBX3 knockdown increased apoptosis in rat bladder carcinoma cells (*7*). TBX3 was also linked to protecting cells against anoikis which is a form of apoptosis that occurs when anchorage dependent cells become detached from the surrounding extracellular matrix and its bypass is associated with epithelial mesenchymal transition (EMT). Indeed, head and neck squamous cell carcinoma (HNSCC) cells depleted of TBX3 underwent anoikis (*7*). The overexpression of TBX3 or TBX3+2a in kidney mesangial cells was also reported to result in the evasion of serum starvation-triggered apoptosis (*48*). Furthermore, silencing Tbx3 in rat bladder carcinoma cells rendered the cells sensitive to doxorubicin-induced apoptosis and the overexpression of TBX3 was associated with a chemotherapy-resistant phenotype (*49*). Similar findings showed that knocking down TBX3 sensitized human colorectal carcinoma cells to doxorubicin *via* activating the p14ARF- p53 pathway (*50*).

A number of studies also support a role for TBX3 in promoting cell cycle progression and proliferation through its ability to disrupt the p19ARF-p53-p21 pathway. Indeed, TBX3 protein and mRNA levels peak in S-phase and chondrosarcoma cells in which TBX3 is depleted accumulate in S-phase with a corresponding increase in p19ARF, p53 and p21 levels (*51*). The

increased levels of TBX3 in S-phase were shown to occur transcriptionally through activation by c-Myc at E-box motifs located at -1210 and -701 bps and post-translationally by cyclin A-CDK2 phosphorylation (*51*). Together these results suggest that TBX3 functions as a pro-proliferative factor, in part, by promoting transition into G2/M. TBX3 can also promote cell proliferation of mammary epithelial cells (MECs) by repressing *19ARF*, which was accompanied by the down-regulation of p21 (*52*). Interestingly, in the same study similar responses were noted in *p53*-null MECs, indicating that the repressive effect of TBX3 on p21 occurred independently of p53. In line with this, depletion of Tbx3 inhibits proliferation of hepatic progenitor cells with a corresponding increase in *p19ARF* mRNA levels and *Tbx3*$^{-/-}$ mouse livers exhibit increased p19ARF and p21 levels although p53 levels remained unaffected (*53*). Importantly, TBX3 can promote cell proliferation by directly binding and repressing the *p21* promoter *via* a consensus T-element which requires the T-box and R2 repression domains (*14,54*). It is interesting to note that TBX2 directly represses the *p21* promoter through the same T-element (*7*). It would therefore be interesting to determine whether TBX2 and TBX3 have redundant pro-proliferative roles in repressing the *p21* promoter in cancers where they are both expressed or whether additional factors, such as post-translational modifications and/or the availability of co-factors, will determine which of the two T-box factors regulates *p21*.

TBX3 may also impinge on other cellular pathways to promote cell cycle progression and proliferation. For example, an inverse correlation between the mRNA and protein levels of TBX3 and the tumour suppressor PTEN was identified in HNSCC cells (*55*). PTEN is a negative regulator of the phosphoinositide 3-kinase/ Protein kinase B (PI3K/AKT) pathway and suppresses oncogenic processes such as proliferation, cell survival and migration (*56*). Importantly, TBX3 can directly repress *PTEN* by binding a 132 bp DNA region within its promoter (*55*). Interestingly, the authors found that this region of the *PTEN* promoter does not contain a T-element and thus speculated that TBX3 may repress *PTEN* by either interacting with transcriptional co-repressors or by interfering with positive activators of *PTEN*. It would be interesting to determine the functional significance of *PTEN* repression by TBX3 especially on cell proliferation, survival and migration.

TBX3 has also been shown to negatively regulate proliferation in support of cell migration, a phenomenon known as the proliferation/migration dichotomy (*57,58*). Knocking down TBX3 by shRNA inhibited migration and enhanced proliferation of breast cancer and melanoma cells which was accompanied by a decrease in the expression of p14ARF, p53 and p21 (*57*). Furthermore, ectopic overexpression of TBX3 in non-invasive WM1650 radial growth phase (RGP) melanoma cells and the upregulation of TBX3 by RA in vertical growth phase (VGP) melanoma cells reduced the proliferative capacity of the cells with the former being associated with an increase in migratory ability (*58,59*). It is important to note that Transforming growth factor-beta (TGF-β) contributes to breast cancer progression by inhibiting cell proliferation and promoting migration and recent studies have shown that TBX3 is required for the anti-proliferative and pro-migratory effects of TGF-β in breast epithelial cells (*60,61*). The mechanism(s) that determines whether TBX3 functions as a pro-proliferative or anti-proliferative factor is not known.

2.3.2. *TBX3 in tumour formation, invasion and metastasis*

TBX3 has also been shown to contribute to tumour formation, migration and invasion (Figure 2, left panel). Indeed, knockdown of TBX3 reduced anchorage-independence of colon and hepatoma carcinoma cell lines and when these cells were engineered to express a Tbx3 mutant, they had significantly reduced ability to form tumours in nude mice (*49*). A study by Chen *et al*. showed that elevated *TBX3* mRNA levels strongly correlated with metastasis in breast cancer and this was similarly observed by Fillmore *et al*. in (ER)-positive breast cancer tumours (*62,63*). Importantly, silencing TBX3 by shRNA inhibited *in vitro* tumour forming ability as well as migration of MCF7 breast cancer cells (*57*). Furthermore, Mowla *et al*. demonstrated that treatment of MCF7 cells with phorbol 12-myristate 13-acetate (PMA) induced TBX3 expression which resulted in increased cell migration (*64*). Several lines of evidence suggest that TBX3 also plays an important role in the transition of non-malignant RGP melanoma to a malignant VGP melanoma. TBX3 levels increase specifically in VGP and metastatic melanoma cells and whereas ectopic overexpression of TBX3 in RGP melanoma cells was sufficient to promote tumour forming ability and invasion, knockdown of TBX3 in VGP cells inhibited migration and tumour formation (*57,58*). The overexpression of TBX3 has also been positively linked to migration and invasion of pancreatic ductal adenocarcinoma (PDAC). Compared to normal adjacent and healthy donors, PDAC tissue samples had increased levels of *TBX3* mRNA and stained positive for TBX3 protein (*65*). Furthermore, *in vitro* assays show that overexpression of TBX3 in Pan1 and BxPC3 pancreatic cell lines significantly enhanced their migratory ability and invasion potential and xenotransplantation experiments revealed that TBX3 overexpressing Pan1 and BxPC3 cells induced significantly larger tumours than their controls. It is interesting to note that the TBX3 overexpressing tumours expressed the angiogenesis associated markers FGF2 and VEGFA which suggests that TBX3 may also be involved in angiogenesis in PDAC. More recently, TBX3 was also linked to tumour formation and migration in several sarcoma subtypes. Ectopic overexpression of

TBX3 in chondrosarcoma cells enhanced their ability to form tumours in mice and knockdown of TBX3 inhibited migration of chondrosarcoma, liposarcoma and rhabdomyosarcoma cells (*44*).

Decreased expression of E-cadherin in several cancers including melanoma, breast, ovarian and non-small cell lung carcinoma have been shown to be associated with tumour invasiveness, metastasis and poor patient prognosis (*66*). There is good evidence to suggest that a key mechanism by which TBX3 promotes these oncogenic processes is through its ability to repress *E-cadherin*. A study by Rodriguez *et al*. showed that increased expression of TBX3 correlated with low levels of E-cadherin in metastatic melanoma tissue samples (*67*). The authors further established using melanoma cells that TBX3 directly binds and represses the *E-cadherin* promoter *in vitro* and *in vivo* through a half consensus T-element close to the transcription initiation site. This repression was shown to be physiologically relevant because when TBX3 was silenced in metastatic melanoma cells, E-cadherin levels increased. Furthermore, TBX3 is transcriptionally indirectly upregulated by the oncoprotein BRAF V600E, which is constitutively activated in 50% of melanomas, and is a substrate and effector of AKT3, which is activated in ~70% of advanced stage melanomas where it plays a critical pro-invasive role (*68,69*). Importantly, TBX3 was shown to repress *E-cadherin* downstream of both these pathways to promote migration and invasion of melanoma cells. Interestingly, phosphorylation of TBX3 by AKT3 in melanoma enhanced its ability to repress *E-cadherin* and phosphorylation of TBX3 by p38 in normal kidney cells decreased E-cadherin protein levels (*70*). This suggests that phosphorylation may play an important role in modulating TBX3 target gene regulation. Elevated levels of TBX3 were also shown to correlate with decreased E-cadherin levels in squamous carcinoma cells (*71*). In addition, work by Du *et al*. revealed that a PKC/TBX3/E-cadherin signalling cascade exists in human bladder cancer cells and that the regulation of *E-cadherin* by TBX3 occurs in a PKC-dependent manner (*72*). It is noteworthy that in sarcomas, the ability of TBX3 to promote migration was not due to *E-cadherin* repression. This suggests that the molecular mechanism(s) underlying the pro-migratory role of TBX3 in different cancers may be different and future studies identifying TBX3 target genes as well as signalling pathways that upregulate TBX3 would likely shed light on this.

2.3.3. *TBX3 and cancer stem cells*

Cancer stem cells (CSCs) are a biologically distinct subpopulation of tumour cells which have indefinite potential for self-renewal and they are thought to ultimately drive tumourigenesis. In addition, CSCs are often not targeted by traditional chemotherapeutic agents and are associated with drug-resistance and clinical relapse after remission (*73*). A study by Fillmore *et al*. revealed that the addition of oestrogen to a number of ERα-positive breast cancer cell lines resulted in a dramatic increase in the number of CSCs which required TBX3 (*63*). Importantly, the knockdown of TBX3 significantly reduced CSC numbers in a variety of breast cancer cell lines and abrogated their ability to form tumourspheres, a spherical formation reported to be generated by the proliferation of a single CSC. On the other hand, TBX3 overexpression resulted in a significant increase in CSC numbers, tumoursphere-forming ability and a more than 100-fold increase in tumour-seeding potential *in vivo*. A recent study by Perkhofer *et al*. also demonstrated that TBX3 overexpressing pancreatic cancer cells had increased sphere-forming ability and that TBX3 induced the CSC phenotype of primary pancreatic cancer cells by regulating NODAL/ACTIVIN signalling through an autocrine positive feedback loop (*65*). In light of this, it would be interesting to determine whether TBX3 impacts similarly on CSCs in other cancers and if this is an additional mechanism through which it contributes to cancer development and anti-cancer drug resistance.

2.3.4. *The tumour suppressor role of TBX3*

In contrast to the large body of work implicating TBX3 as an oncoprotein, there are also studies that have implicated it as a tumour suppressor (Figure 2, right panel). Using microarray analyses, Lyng *et al*. demonstrated that *TBX3* gene expression is downregulated in uterine cervical cancer samples, which strongly correlated with lymph node metastasis and reduced progression-free patient survival (*74*). Subsequent studies have also shown that *TBX3* levels are epigenetically silenced by methylation in glioblastoma, gastric, bladder and prostate cancer (*7*). Yamashita *et al*. examined methylation-silenced genes in gastric cancer and revealed that the *TBX3* promoter is methylated in at least one primary gastric cancer, but not normal gastric mucosa (*75*). TBX3 methylation was subsequently revealed to be associated with a significantly lowered survival rate in a cohort of glioblastoma patients (*76*). Following this, White-al Habeeb *et al*. demonstrated that *TBX3* is differentially methylated in Gleason score (GS) 8 vs GS6 prostate cancer tumour samples, with increased methylation occurring in the more aggressive GS8 samples (*77*). Collectively these studies suggest that under certain circumstances TBX3 suppression may promote cancer progression. Furthermore, a study investigating the genome-wide methylation pattern of fresh-frozen bladder cancer tumour tissue revealed that *TBX3* methylation is associated with the progression of non-muscle invasive human bladder tumours (Pta) to muscle-invasive tumours (MI) (*78*). The authors of this study also showed that *TBX3* methylation was an

independent predictor of bladder cancer progression and that it correlated with reduced progression-free survival. In a follow-up study, the same group performed methylation analyses on formalin-fixed paraffin-embedded tumour tissue from 192 patients with Pta, 40% of whom experienced progression to MI (*79*). They demonstrated that patients with low *TBX3* methylation experienced significantly increased progression-free survival and that, within 10 years of follow-up time, *TBX3* was an independent prognostic marker of progression risk. Interestingly, both studies revealed that methylation of the *TBX3* homologue, *TBX2*, was also linked to tumour progression of bladder cancer (*78,79*).

The above studies together suggested that TBX3 may function as a tumour suppressor and a comprehensive characterisation of the tumour suppressor role of TBX3 was recently provided by Willmer *et al*. (*44*). The authors showed that the TBX3 protein is upregulated in a number of transformed fibroblast and fibrosarcoma cell lines and tissues. Using TBX3 overexpressing and knock down fibrosarcoma cell culture models they demonstrated that TBX3 discourages substrate- dependent and -independent cell proliferation, migration and tumour formation. Importantly, whereas the knockdown of TBX3 was able to significantly increase the *in vivo* tumour forming ability of fibrosarcoma cells in a mouse model, TBX3 overexpressing cells formed smaller tumours in the same model. These results demonstrate that TBX3 behaves as a tumour suppressor in transformed fibroblast and fibrosarcoma cells, suggesting it may function as either oncoprotein or tumour suppressor depending on cellular context. It will be important to elucidate the molecular mechanism(s) that enables it to switch between these functions.

2.4. *Signalling pathways that regulate TBX3 expression in cancer*

Despite the catastrophic effects observed when TBX3 expression is deregulated very little is still known about the signalling pathways that regulate TBX3 levels. In the last few years some developmental pathways, that are important in cancer, have been implicated and these will be discussed below and are indicated in Figure 2.

2.4.1. *Wnt/β-catenin signalling pathway*

The canonical Wnt/β-catenin pathway plays critical roles during embryogenesis and adult tissue homeostasis, where it regulates a number of key processes including proliferation, differentiation and cell fate determination (*80*). Aberrant activation of the Wnt/β-catenin pathway, often caused by constitutive activation of β-catenin, has been implicated in a number of cancers and Renard *et al*. have suggested that TBX3 is a direct target of this pathway in liver tumourigenesis (*49*). Using microarray analyses, they demonstrated that *TBX3* is upregulated in mouse hepatocellular carcinoma (HCC) tumour samples carrying an active mutant form of β-catenin. In addition, the authors revealed a correlation of increased *TBX3* expression with a mutant active form of β-catenin in human and mouse HCC and human hepatoblastoma. Furthermore, they demonstrated that β-catenin, in complex with its co-activator Tcf, directly activated the *TBX3* promoter through a Tcf-binding element, resulting in enhanced proliferation, tumour formation, as well as protection from doxorubicin-induced apoptosis. The authors concluded that TBX3 is a key mediator of the proliferation and survival activities of β-catenin in liver tumourigenesis.

Following this, Lachenmayer *et al*. investigated the effect of the multi-kinase inhibitor Sorafenib, the only Food and Drug Administration (FDA) approved drug for HCC treatment, on the human HepG2 HCC cell line (*81*). They showed that Sorafenib inhibits β-catenin levels with a corresponding reduction in *TBX3* mRNA levels. In addition, one of the oldest and best known Chinese herbal medicines, rhubarb root, is used to treat liver disease and exhibits anti-cancer properties through the induction of apoptosis in a number of cancers (*82*). Tsai *et al*. found that it also attenuates Wnt/β-catenin signalling in human HCC cells which was accompanied by decreased TBX3 protein levels (*82*).

Interestingly, a study by Cavard *et al*. suggests that the regulation of TBX3 by the Wnt/β-catenin pathway may not be limited to liver cancer (*83*). They examined gene expression profiles in solid pseudopapillary pancreatic neoplasms where the Wnt/β-catenin pathway is aberrantly activated and demonstrated that *TBX3* mRNA is overexpressed. The interplay between Wnt signalling and TBX3 was also reported by Arendt *et al*., who showed that both Wnt and TBX3 could regulate proliferation of human breast progenitor cells and that TBX3 induced autocrine Wnt signalling in these cells, possibly *via* a feedback loop (*84*).

2.4.2. *Fibroblast growth factor (FGF) signalling pathway*

FGF signalling has been shown to be important for the regulation of cell proliferation, survival, migration as well as differentiation, and deregulated FGF signalling is important for the pathogenesis of a number of cancers (*85*). It is well established that TBX3 regulates this signalling pathway during mammary gland development and it has also been suggested that TBX3 lies downstream of FGF (*86*). Fillmore et al. demonstrated that oestrogen and FGF signalling upregulated the expression of TBX3 in breast cancer (*63*). Treatment of ERα-positive breast cancer cell lines with oestrogen resulted in a substantial increase in secreted FGF9, which in turn resulted in significantly increased TBX3 mRNA and protein levels. Importantly, when cells were treated with oestrogen and an FGF9 inhibitor this was decreased, while treatment with both oestrogen and FGF9 resulted

in an even greater increase in TBX3 mRNA and protein expression. Similarly, when ERα-negative breast cancer cell lines were treated with FGF9, there was a marked increase in TBX3 protein, suggesting that this regulation is not specific to ERα-positive breast cancer. Taken together, these findings suggest that FGF signalling mediates the upregulation of TBX3 by oestrogen in breast cancer and, as mentioned previously, promotes the expansion of breast CSCs.

2.4.3. *Protein kinase C (PKC) signalling pathway*

The PKC family consists of phospholipid-dependent serine/threonine-specific protein kinases which mediate various cellular functions including proliferation, apoptosis and differentiation (*87*). The deregulation of PKC signalling has been implicated in cancer, including breast cancer where it promotes tumourigenesis by modulating cell proliferation, migration, apoptosis and survival (*87*). Many studies which have investigated the role of the PKC pathway have employed the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA, also called PMA) which activates PKC isoforms by translocating them to specific cellular compartments (*87*). Mowla *et al*. showed that in prostate epithelial and lung fibroblast cells, PMA, increased TBX3 mRNA and protein in a PKC-dependent manner (*64*). Furthermore, they demonstrated that the activator protein-1 (AP-1) transcription factors, c-Jun and JunB, bound a degenerate PMA response element in the *TBX3* promoter, mediating its upregulation. Finally, as mentioned previously, they showed that TBX3 mediated the pro-migratory effect of PMA in MCF7 breast cancer cells. The regulation of TBX3 by the PKC pathway has been further supported by Du *et al*. who showed that knocking down Phospholipase C epsilon (PLCε) in bladder cancer cell lines resulted in a corresponding inhibition of PKCα/β, which in turn reduced TBX3 mRNA and protein levels (*72*). Importantly, they also demonstrated that in bladder cancer the repression of *E-cadherin* by PKCα/β signalling is mediated by TBX3.

2.4.4. *Retinoic acid (RA) signalling pathway*

All-trans RA, a metabolite of vitamin A (all-trans-retinol), plays an important role in embryo development, where it regulates gene transcription to modulate a number of biological processes, including cell proliferation, differentiation and apoptosis (*88*). Compromised RA signalling often occurs early in tumourigenesis, including in breast cancer (*89*). Initial studies provided a preliminary indication that *TBX3* may be regulated by the RA pathway in the development of chick and mouse limbs (*90,91*). Ballim *et al*. confirmed this regulation during mouse limb development and expanded these findings to show that RA treatment of the ME1402 VGP melanoma cell line resulted in a substantial increase in TBX3 mRNA and protein, and a correlative decrease in proliferation (*59*). Furthermore, knockdown of TBX3 expression by a shRNA approach significantly reduced the growth-inhibitory effect of RA. Finally, the authors demonstrated that RA transcriptionally activates *TBX3* through directly binding a degenerate RA response element half site at -87 bp. Together these results suggest that TBX3 partly mediates the RA-regulated inhibition of cell proliferation in the human melanoma cell line tested.

2.4.5. *Transforming growth factor-beta 1 (TGF-β1) signalling pathway*

The TGF-β growth factor cytokine family is composed of a large number of secreted polypeptides that activate cellular responses involved in development, homeostasis and the immune system (*92*). In addition, TGF-β1 signalling has been widely reported to have a dual role in the progression of cancer. While it acts as a tumour suppressor and inhibits cell proliferation during the early stages of carcinogenesis, it promotes migration and metastasis in the late stages of the disease (*92*). Similar to TBX3, TGF-β1 signalling is critical for the development of the mammary glands but also contributes to breast cancer progression through the inhibition of cell proliferation and promotion of migration (*93*). Importantly, Li *et al*. demonstrated that TGF-β1 treatment transcriptionally upregulated *TBX3* in breast epithelial and keratinocyte cells (*61*). They showed that JunB and Smad4 mediate this effect and directly bind to the *TBX3* promoter at a degenerate Smad Binding Element (SBE). The authors also revealed that TBX3 mediated the anti-proliferative and pro-migratory effects of TGF-β1 in these cells. In a follow-up paper they demonstrated that this anti-proliferative function involves the ability of TBX3 to directly bind and repress the pro-proliferative factor *TBX2* (*60*). Finally, they showed that the TGF-β1/TBX3/TBX2 axis resulted in the upregulation of *p21WAF1*, leading to cell cycle inhibition. Recent work by Wensing & Campos also showed that TGF-β1 treatment of immortalised human mesangial cells resulted in a substantial increase in *TBX3* and *TBX3+2a* mRNA which resulted in the evasion of serum starvation- triggered apoptosis (*48*). These findings suggest that TGF-β1 may upregulate TBX3 to simultaneously inhibit proliferation but bypass apoptosis. However, more work is needed to explore this further.

2.4.6. *Phosphatidylinositol 3-kinase/Protein kinase B (PI3K/Akt) signalling pathway*

PI3Ks constitute a lipid kinase family that activate the serine/threonine kinases, Akt1, Akt2 and Akt3. These highly homologous isoforms phosphorylate a plethora of substrates that are involved in the regulation of key cellular processes including cell proliferation, apoptosis, and migration (*94*). It is well established that constitutive

activation of Akt, through *PI3K* amplification or *PTEN* mutation, contributes to the oncogenic process and is associated with a poor prognosis and resistance to chemo- and radio-therapy (*94*). Indeed, Akt activation is one of the most frequent molecular alterations in cancers, including melanoma, gastric, pancreatic, breast, sarcoma and prostate cancer (*95-98*). While there are instances where the three Akt isoforms are able to compensate for each other, recent research show that their tissue expression patterns and functions are different (*99,100*). The PI3K/Akt pathway is activated in 70% of all melanoma cases and Peres *et al*. reported that TBX3 is a substrate and effector of this pathway in melanoma (*69*). Consistent with other reports, the authors demonstrated that Akt3 is the predominant isoform activated in a panel of human melanoma cell lines. Furthermore, Akt3 post-translationally upregulated TBX3 expression through phosphorylation at S720 in ME1402 and MM200 VGP melanoma cells, which promoted protein stabilisation. Lastly, they demonstrated that TBX3 mediated the oncogenic activity of the PI3K/Akt pathway in these cells, as phosphorylation of TBX3 at this site resulted in increased nuclear localisation of TBX3 protein, repression of E-cadherin and promotion of migration and invasion.

3. Conclusion

The transcription factor TBX3 provides an important link between embryonic development and cancer. While mutations resulting in decreased levels of TBX3 lead to ulnar-mammary syndrome, increased levels of TBX3 are linked to several epithelial derived cancers and a diverse subset of soft tissue and bone sarcomas. Indeed, TBX3 is a key driver of a number of cancer processes including proliferation, tumour formation, invasion and metastasis and it has therefore been proposed as an important novel molecular target for anti-cancer treatments. There is also evidence that in certain cellular contexts TBX3 may function as a brake to prevent tumour progression. However, there is still very little known about the molecular mechanisms regulating the tumour promoting and tumour suppressor functions of TBX3 and what enables it to switch between these two roles. This information has important implications for targeting TBX3 in anti-cancer drugs especially since directly targeting TBX3 is unlikely to be a viable option. This could involve the following three approaches either alone or in combination: targeting signalling pathways required for TBX3 overexpression; targeting the enzymatic activities of TBX3 co-factors; and targeting the enzymatic activity of genes that encode enzymes that are activated by TBX3.

Acknowledgements

This project was supported by grants from the SA Medical Research Council, National Research Foundation (NRF), Cancer Association of South Africa (CANSA), the University of Cape Town and the Harry Crossley foundation.

References

1. Papaioannou VE. The *T-box* gene family: Emerging roles in development, stem cells and cancer. Development. 2014; 141:3819-3833.
2. Wilson V, Conlon FL. The T-box family. Genome Biol. 2002; 3:3008.1-3008.7
3. Lingbeek ME, Jacobs JJ, van Lohuizen M. The T-box repressors TBX2 and TBX3 specifically regulate the tumor suppressor gene *p14ARF via* a variant T-site in the initiator. J Biol Chem. 2002; 277:26120-26127.
4. van den Boogaard M, Wong LY, Tessadori F, Bakker ML, Dreizehnter LK, Wakker V, Bezzina CR, t Hoen PA, Bakkers J, Barnett P, Christoffels VM. Genetic variation in T-box binding element functionally affects SCN5A/SCN10A enhancer. J Clin Invest. 2012; 122:2519-2530.
5. Papaioannou VE. *T-box* genes in development: From hydra to humans. Int Rev Cytol. 2001; 207:1-70.
6. Packham EA, Brook JD. *T-box* genes in human disorders. Hum Mol Genet. 2003; 12:R37-R44.
7. Wansleben S, Peres J, Hare S, Goding CR, Prince S. T-box transcription factors in cancer biology. Biochim Biophys Acta. 2014; 1846:380-391.
8. Agulnik SI, Garvey N, Hancock S, Ruvinsky I, Chapman DL, Agulnik I, Bollag R, Papaioannou V, Silver LM. Evolution of mouse *T-box* genes by tandem duplication and cluster dispersion. Genetics. 1996; 144:249-254.
9. Bamshad M, Lin RC, Law DJ, Watkins WC, Krakowiak PA, Moore ME, Franceschini P, Lala R, Holmes LB, Gebuhr TC, Bruneau BG, Schinzel A, Seidman JG, Seidman CE, Jorde LB. Mutations in human TBX3 alter limb, apocrine and genital development in ulnar-mammary syndrome. Nat Gen. 1997; 16:311-315.
10. Campbell C, Goodrich K, Casey G, Beatty B. Cloning and Mapping of a Human Gene (*TBX2*) Sharing a Highly Conserved Protein Motif with the Drosophila omb Gene. Genomics. 1995; 28:255-260.
11. Law DJ, Gebuhr T, Garvey N, Agulnik SI, Silver LM. Identification, characterization and localization to chromosome 17q21-22 of the human TBX2 homolog, member of a conserved developmental gene family. Mamm Genome. 1995; 6:793-797.
12. Fan W, Huang X, Chen C, Gray J, Huang T. TBX3 and its isoform TBX3+2a are functionally distinctive in inhibition of senescence and are overexpressed in a subset of breast cancer cell lines. Cancer Res. 2004; 64:5132-5139.
13. Bamshad M, Le T, Watkins WS, et al. The spectrum of mutations in TBX3: Genotype/Phenotype relationship in ulnar-mammary syndrome. Am J Hum Genet. 1999; 64:1550-1562.
14. Hoogaars WM, Barnett P, Rodriguez M, Clout DE, Moorman AF, Goding CR, Christoffels VM. TBX3 and its splice variant TBX3 + exon 2a are functionally similar. Pigment Cell Melanoma Res. 2008; 21:379-387.
15. Zhao D, Wu Y, Chen K. Tbx3 isoforms are involved in pluripotency maintaining through distinct regulation of Nanog transcriptional activity. Biochem Biophys Res Commun. 2014; 444:411-414.
16. Kumar PP, Franklin S, Emechebe U, Hu H, Moore B,

Lehman C, Yandell M, Moon AM. TBX3 regulates splicing *in vivo*: A novel molecular mechanism for Ulnar-mammary syndrome. PLoS Genet. 2014; 10:e1004247.
17. Carlson H, Ota S, Campbell CE, Hurlin PJ. A dominant repression domain in Tbx3 mediates transcriptional repression and cell immortalization: Relevance to mutations in Tbx3 that cause ulnar-mammary syndrome. Hum Mol Genet. 2001; 10:2403-2413.
18. Boogerd CJJ, Wong LYE, van den Boogaard M, Bakker ML, Tessadori F, Bakkers J, 't Hoen PAC, Moorman AF, Christoffels VM, Barnett P. Sox4 mediates Tbx3 transcriptional regulation of the gap junction protein Cx43. Cell Mol Life Sci. 2011; 68:3949-3961.
19. Lu R, Yang A, Jin Y. Dual Functions of T-Box 3 (Tbx3) in the Control of Self-renewal and Extraembryonic Endoderm Differentiation in Mouse Embryonic Stem Cells. J Biol Chem. 2011; 286:8425-8436.
20. Coll M, Seidman JG, Muller CW. Structure of the DNA-bound T-box domain of human TBX3, a transcription factor responsible for ulnar-mammary syndrome. Structure. 2002; 10:343-356.
21. Lu J, Li X-P, Dong Q, Kung H-f, He M-L. TBX2 and TBX3: The special value for anticancer drug targets. Biochim Biophys Acta. 2010; 1806:268-274.
22. Kumar PP, Emechebe U, Smith R, Franklin S, Moore B, Yandell M, Lessnick SL, Moon AM. Coordinated control of senescence by lncRNA and a novel T-box3 co-repressor complex. Elife. 2014; 3.
23. Chapman DL, Garvey N, Hancock S, Alexiou M, Agulnik SI, Gibson-Brown JJ, Cebra-Thomas J, Bollag RJ, Silver LM, Papaioannou VE. Expression of the T-box family genes, Tbx1-Tbx5, during early mouse development. Dev Dyn. 1996; 206:379-390.
24. Washkowitz AJ, Gavrilov S, Begum S, Papaioannou VE. Diverse functional networks of Tbx3 in development and disease. Wiley Interdiscip Rev Syst Biol Med. 2012; 4:273-283.
25. Davenport TG, Jerome-Majewska LA, Papaioannou VE. Mammary gland, limb and yolk sac defects in mice lacking *Tbx3*, the gene mutated in human ulnar mammary syndrome. Development. 2003; 130:2263-2273.
26. Jerome-Majewska LA, Jenkins GP, Ernstoff E, Zindy F, Sherr CJ, Papaioannou VE. *Tbx3*, the ulnar-mammary syndrome gene, and Tbx2 interact in mammary gland development through a p19Arf/p53-independent pathway. Dev Dyn. 2005; 234:922-933.
27. Bishop AE, Buttery LD, Polak JM. Embryonic stem cells. J Pathol. 2002; 197:424-429.
28. Ivanova N, Dobrin R, Lu R, Kotenko I, Levorse J, DeCoste C, Schafer X, Lun Y, Lemischka IR. Dissecting self-renewal in stem cells with RNA interference. Nature. 2006; 442:533-538.
29. Niwa H, Ogawa K, Shimosato D, Adachi K. A parallel circuit of LIF signalling pathways maintains pluripotency of mouse ES cells. Nature. 2009; 460:118-122.
30. Jiang K, Ren C, Nair VD. MicroRNA-137 represses Klf4 and Tbx3 during differentiation of mouse embryonic stem cells. Stem Cell Res. 2013; 11:1299-1313.
31. Cioffi M, Vallespinos-Serrano M, Trabulo SM, et al. MiR-93 Controls Adiposity *via* Inhibition of Sirt7 and Tbx3. Cell Rep. 2015; 12:1594-1605.
32. Cheng X, Ying L, Lu L, Galvão AM, Mills JA, Lin HC, Kotton DN, Shen SS, Nostro MC, Choi JK, Weiss MJ, French DL, Gadue P. Self-Renewing Endodermal Progenitor Lines Generated from Human Pluripotent Stem Cells. Cell Stem Cell. 2012; 10:371-384.
33. Kartikasari AE, Zhou JX, Kanji MS, Chan DN, Sinha A, Grapin-Botton A, Magnuson MA, Lowry WE, Bhushan A. The histone demethylase Jmjd3 sequentially associates with the transcription factors Tbx3 and Eomes to drive endoderm differentiation. Embo J. 2013; 32:1393-1408.
34. Price FD, Yin H, Jones A, van Ijcken W, Grosveld F, Rudnicki MA. Canonical Wnt signaling induces a primitive endoderm metastable state in mouse embryonic stem cells. Stem Cells. 2013; 31:752-764.
35. Waghray A, Saiz N, Jayaprakash AD, Freire AG, Papatsenko D, Pereira CF, Lee DF, Brosh R, Chang B, Darr H, Gingold J, Kelley K, Schaniel C, Hadjantonakis AK, Lemischka IR. Tbx3 Controls Dppa3 Levels and Exit from Pluripotency toward Mesoderm. Stem Cell Reports. 2015; 5:97-110.
36. Han J, Yuan P, Yang H, Zhang J, Soh BS, Li P, Lim SL, Cao S, Tay J, Orlov YL, Lufkin T, Ng H-H, Tam W-L, Lim B. Tbx3 improves the germ-line competency of induced pluripotent stem cells. Nature. 2010; 463:1096-1100.
37. Declercq J, Sheshadri P, Verfaillie CM, Kumar A. Zic3 enhances the generation of mouse induced pluripotent stem cells. Stem Cells Dev. 2013; 22:2017-2025.
38. Bakker ML, Boukens BJ, Mommersteeg MT, Brons JF, Wakker V, Moorman AF, Christoffels VM. Transcription factor Tbx3 is required for the specification of the atrioventricular conduction system. Circ Res. 2008; 102:1340-1349.
39. Falco G, Lee SL, Stanghellini I, Bassey UC, Hamatani T, Ko MS. *Zscan4*: A novel gene expressed exclusively in late 2-cell embryos and embryonic stem cells. Dev Biol. 2007; 307:539-550.
40. Dan J, Li M, Yang J, Li J, Okuka M, Ye X, Liu L. Roles for Tbx3 in regulation of two-cell state and telomere elongation in mouse ES cells. Sci Rep. 2013; 3:3492.
41. Esmailpour T, Huang T. TBX3 promotes human embryonic stem cell proliferation and neuroepithelial differentiation in a differentiation stage-dependent manner. Stem Cells. 2012; 30:2152-2163.
42. Weinberger L, Ayyash M, Novershtern N, Hanna JH. Dynamic stem cell states: Naive to primed pluripotency in rodents and humans. Nat Rev Mol Cell Bio. 2016; 17:155-169
43. Miao ZF, Liu XY, Xu HM, Wang ZN, Zhao TT, Song YX, Xing YN, Huang JY, Zhang JY, Xu H, Xu YY. Tbx3 overexpression in human gastric cancer is correlated with advanced tumor stage and nodal status and promotes cancer cell growth and invasion. Virchows Archiv. 2016; 469:505-513.
44. Willmer T, Cooper A, Sims D, Govender D, Prince S. The T-box transcription factor 3 is a promising biomarker and a key regulator of the oncogenic phenotype of a diverse range of sarcoma subtypes. Oncogenesis. 2016; 5:e199.
45. Yarosh W, Barrientos T, Esmailpour T, Lin L, Carpenter PM, Osann K, Anton-Culver H, Huang T. TBX3 is overexpressed in breast cancer and represses p14 ARF by interacting with histone deacetylases. Cancer Res. 2008; 68:693-699.
46. Elmore S. Apoptosis: A Review of Programmed Cell Death. Toxicol Pathol. 2007; 35:495-516.
47. Carlson H, Ota S, Song Y, Chen Y, Hurlin PJ. Tbx3 impinges on the p53 pathway to suppress apoptosis, facilitate cell transformation and block myogenic differentiation. Oncogene. 2002; 21:3827-3835.

48. Wensing LA, Campos AH. TBX3, a downstream target of TGF-beta1, inhibits mesangial cell apoptosis. Exp Cell Res. 2014; 328:340-350.
49. Renard CA, Labalette C, Armengol C, Cougot D, Wei Y, Cairo S, Pineau P, Neuveut C, de Reynies A, Dejean A, Perret C, Buendia MA. Tbx3 is a downstream target of the Wnt/beta-catenin pathway and a critical mediator of beta-catenin survival functions in liver cancer. Cancer Res. 2007; 67:901-910.
50. Zhang JF, He ML, Qi D, Xie WD, Chen YC, Lin MC, Leung PC, Zhang YO, Kung HF. Aqueous extracts of Fructus Ligustri Lucidi enhance the sensitivity of human colorectal carcinoma DLD-1 cells to doxorubicin-induced apoptosis *via* Tbx3 suppression. Integr Cancer Ther. 2011; 10:85-91.
51. Willmer T, Peres J, Mowla S, Abrahams A, Prince S. The T-Box factor TBX3 is important in S-phase and is regulated by c-Myc and cyclin A-CDK2. Cell Cycle. 2015; 14:3173-3183.
52. Platonova N, Scotti M, Babich P, Bertoli G, Mento E, Meneghini V, Egeo A, Zucchi I, Merlo GR. *TBX3*, the gene mutated in ulnar-mammary syndrome, promotes growth of mammary epithelial cells *via* repression of p19ARF, independently of p53. Cell Tissue Res. 2007; 328:301-316.
53. Suzuki A, Sekiya S, Buscher D, Izpisua Belmonte JC, Taniguchi H. Tbx3 controls the fate of hepatic progenitor cells in liver development by suppressing p19ARF expression. Development. 2008; 135:1589-1595.
54. Willmer T, Hare S, Peres J, Prince S. The T-box transcription factor TBX3 drives proliferation by direct repression of the p21WAF1 cyclin-dependent kinase inhibitor. Cell Division. 2016; 11:6.
55. Burgucu D, Guney K, Sahinturk D, Ozbudak IH, Ozel D, Ozbilim G, Yavuzer U. Tbx3 represses PTEN and is over-expressed in head and neck squamous cell carcinoma. BMC Cancer. 2012; 12:481.
56. Chalhoub N, Baker SJ. PTEN and the PI3-kinase pathway in cancer. Annu Rev Pathol. 2009; 4:127-150.
57. Peres J, Davis E, Mowla S, Bennett DC, Li JA, Wansleben S, Prince S. The Highly Homologous T-Box Transcription Factors, TBX2 and TBX3, Have Distinct Roles in the Oncogenic Process. Genes Cancer. 2010; 1:272-282.
58. Peres J, Prince S. The T-box transcription factor, TBX3, is sufficient to promote melanoma formation and invasion. Mol Cancer. 2013; 12:117-117.
59. Ballim RD, Mendelsohn C, Papaioannou VE, Prince S. The ulnar-mammary syndrome gene, *Tbx3*, is a direct target of the retinoic acid signaling pathway, which regulates its expression during mouse limb development. Mol Bio Cell. 2012; 23:2362-2372.
60. Li J, Ballim D, Rodriguez M, Cui R, Goding CR, Teng H, Prince S. The Anti-proliferative Function of the TGF-β1 Signaling Pathway Involves the Repression of the Oncogenic TBX2 by Its Homologue TBX3. J Biol Chem. 2014; 289:35633-35643.
61. Li J, Weinberg MS, Zerbini L, Prince S. The oncogenic TBX3 is a downstream target and mediator of the TGF-β1 signaling pathway. Mol Bio Cell. 2013; 24:3569-3576.
62. Chen ZH, Lu GM, Ji TH. Expression of TBX3 mRNA and its role in the pathogenesis and metastasis of breast cancer. Nan Fang Yi Ke Da Xue Xue Bao. 2009; 29:87-89 (in Chinese).
63. Fillmore CM, Gupta PB, Rudnick JA, Caballero S, Keller PJ, Lander ES, Kuperwasser C. Estrogen expands breast cancer stem-like cells through paracrine FGF/Tbx3 signaling. Proc Natl Acad Sci U S A. 2010; 107:21737-21742.
64. Mowla S, Pinnock R, Leaner VD, Goding CR, Prince S. PMA-induced up-regulation of TBX3 is mediated by AP-1 and contributes to breast cancer cell migration. Biochem J. 2011; 433:145-153.
65. Perkhofer L, Walter K, Costa IG, et al. Tbx3 fosters pancreatic cancer growth by increased angiogenesis and activin/nodal-dependent induction of stemness. Stem Cell Res. 2016; 17:367-378.
66. Canel M, Serrels A, Frame MC, Brunton VG. E-cadherin–integrin crosstalk in cancer invasion and metastasis. J Cell Sci. 2013; 126:393-401.
67. Rodriguez M, Aladowicz E, Lanfrancone L, Goding CR. Tbx3 Represses E-Cadherin Expression and Enhances Melanoma Invasiveness. Cancer Res. 2008; 68:7872-7881.
68. Boyd SC, Mijatov B, Pupo GM, Tran SL, Gowrishankar K, Shaw HM, Goding CR, Scolyer RA, Mann GJ, Kefford RF, Rizos H, Becker TM. Oncogenic B-RAF(V600E) signaling induces the T-Box3 transcriptional repressor to repress E-cadherin and enhance melanoma cell invasion. J Invest Dermatol. 2013; 133:1269-1277.
69. Peres J, Mowla S, Prince S. The T-box transcription factor, TBX3, is a key substrate of AKT3 in melanomagenesis. Oncotarget. 2015; 6:1821-1833.
70. Yano T, Yamazaki Y, Adachi M, Okawa K, Fort P, Uji M, Tsukita S, Tsukita S. Tara up-regulates E-cadherin transcription by binding to the Trio RhoGEF and inhibiting Rac signaling. J Cell Biol. 2011; 193:319-332.
71. Humtsoe JO, Koya E, Pham E, Aramoto T, Zuo J, Ishikawa T, Kramer RH. Transcriptional profiling identifies upregulated genes following induction of epithelial-mesenchymal transition in squamous carcinoma cells. Exp Cell Res. 2012; 318:379-390.
72. Du HF, Ou LP, Yang X, Song XD, Fan YR, Tan B, Luo CL, Wu XH. A new PKCalpha/beta/TBX3/E-cadherin pathway is involved in PLCepsilon-regulated invasion and migration in human bladder cancer cells. Cell Signal. 2014; 26:580-593.
73. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. Nat Rev Cancer. 2005; 5:275-284.
74. Lyng H, Brovig RS, Svendsrud DH, Holm R, Kaalhus O, Knutstad K, Oksefjell H, Sundfor K, Kristensen GB, Stokke T. Gene expressions and copy numbers associated with metastatic phenotypes of uterine cervical cancer. BMC Genomics. 2006; 7:268.
75. Yamashita S, Tsujino Y, Moriguchi K, Tatematsu M, Ushijima T. Chemical genomic screening for methylation-silenced genes in gastric cancer cell lines using 5-aza-2'-deoxycytidine treatment and oligonucleotide microarray. Cancer Sci. 2006; 97:64-71.
76. Etcheverry A, Aubry M, de Tayrac M, Vauleon E, Boniface R, Guenot F, Saikali S, Hamlat A, Riffaud L, Menei P, Quillien V, Mosser J. DNA methylation in glioblastoma: Impact on gene expression and clinical outcome. BMC Genomics. 2010; 11:701.
77. White-Al Habeeb NMA, Ho LT, Olkhov-Mitsel E, Kron K, Pethe V, Lehman M, Jovanovic L, Fleshner N, van der Kwast T, Nelson CC, Bapat B. Integrated analysis of epigenomic and genomic changes by DNA methylation dependent mechanisms provides potential novel biomarkers for prostate cancer. Oncotarget. 2014; 5:7858-7869.

78. Kandimalla R, van Tilborg AA, Kompier LC, Stumpel DJ, Stam RW, Bangma CH, Zwarthoff EC. Genome-wide analysis of CpG island methylation in bladder cancer identified TBX2, TBX3, GATA2, and ZIC4 as pTa-specific prognostic markers. Eur Urol. 2012; 61:1245-1256.
79. Beukers W, Kandimalla R, Masius RG, Vermeij M, Kranse R, van Leenders GJ, Zwarthoff EC. Stratification based on methylation of TBX2 and TBX3 into three molecular grades predicts progression in patients with pTa-bladder cancer. Mod Pathol . 2015; 28:515-522.
80. Logan CY, Nusse R. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol. 2004; 20:781-810.
81. Lachenmayer A, Alsinet C, Savic R, et al. Wnt-pathway activation in two molecular classes of hepatocellular carcinoma and experimental modulation by sorafenib. Clin Cancer Res. 2012; 18:4997-5007.
82. Tsai KH, Hsien HH, Chen LM, Ting WJ, Yang YS, Kuo CH, Tsai CH, Tsai FJ, Tsai HJ, Huang CY. Rhubarb inhibits hepatocellular carcinoma cell metastasis *via* GSK-3-beta activation to enhance protein degradation and attenuate nuclear translocation of beta-catenin. Food Chem. 2013; 138:278-285.
83. Cavard C, Audebourg A, Letourneur F, Audard V, Beuvon F, Cagnard N, Radenen B, Varlet P, Vacher-Lavenu MC, Perret C, Terris B. Gene expression profiling provides insights into the pathways involved in solid pseudopapillary neoplasm of the pancreas. J Pathol. 2009; 218:201-209.
84. Arendt LM, St. Laurent J, Wronski A, Caballero S, Lyle SR, Naber SP, Kuperwasser C. Human breast progenitor cell numbers are regulated by WNT and TBX3. PLoS One. 2014; 9:e111442.
85. Katoh M, Nakagama H. FGF receptors: Cancer biology and therapeutics. Med Res Rev. 2014; 34:280-300.
86. Eblaghie MC, Song S-J, Kim J-Y, Akita K, Tickle C, Jung H-S. Interactions between FGF and Wnt signals and Tbx3 gene expression in mammary gland initiation in mouse embryos. J Anat. 2004; 205:1-13.
87. Marengo B, De Ciucis C, Ricciarelli R, Pronzato MA, Marinari UM, Domenicotti C. Protein Kinase C: An attractive target for cancer therapy. Cancers. 2011; 3:531-567.
88. Das BC, Thapa P, Karki R, Das S, Mahapatra S, Liu TC, Torregroza I, Wallace DP, Kambhampati S, Van Veldhuizen P, Verma A, Ray SK, Evans T. Retinoic acid signaling pathways in development and diseases. Bioorg Med Chem. 2014; 22:673-683.
89. Garattini E, Paroni G, Terao M. Retinoids and breast cancer: New clues to increase their activity and selectivity. Breast Cancer Res. 2012; 14:111-111.
90. Suzuki T, Takeuchi J, Koshiba-Takeuchi K, Ogura T. Tbx Genes Specify Posterior Digit Identity through Shh and BMP Signaling. Dev Cell. 2004; 6:43-53.
91. Tumpel S, Sanz-Ezquerro JJ, Isaac A, Eblaghie MC, Dobson J, Tickle C. Regulation of Tbx3 expression by anteroposterior signalling in vertebrate limb development. Dev Biol. 2002; 250:251-262.
92. Imamura T, Hikita A, Inoue Y. The roles of TGF-beta signaling in carcinogenesis and breast cancer metastasis. Breast Cancer. 2012; 19:118-124.
93. Tian F, DaCosta Byfield S, Parks WT, Yoo S, Felici A, Tang B, Piek E, Wakefield LM, Roberts AB. Reduction in Smad2/3 signaling enhances tumorigenesis but suppresses metastasis of breast cancer cell lines. Cancer Res. 2003; 63:8284-8292.
94. Nitulescu GM, Margina D, Juzenas P, Peng Q, Olaru OT, Saloustros E, Fenga C, Spandidos D, Libra M, Tsatsakis AM. Akt inhibitors in cancer treatment: The long journey from drug discovery to clinical use. Int J Oncol. 2016; 48:869-885.
95. Samuels Y, Ericson K. Oncogenic PI3K and its role in cancer. Current Opinion in Oncology. 2006; 18:77-82.
96. Yuan TL, Cantley LC. PI3K pathway alterations in cancer: Variations on a theme. Oncogene. 2008; 27:5497-5510.
97. Tomita Y, Morooka T, Hoshida Y, Zhang B, Qiu Y, Nakamichi I, Hamada K, Ueda T, Naka N, Kudawara I, Aozasa K. Prognostic significance of activated AKT expression in soft-tissue sarcoma. Clin Cancer Res. 2006; 12:3070-3077.
98. Valkov A, Kilvaer TK, Sorbye SW, Donnem T, Smeland E, Bremnes RM, Busund L-T. The prognostic impact of Akt isoforms, PI3K and PTEN related to female steroid hormone receptors in soft tissue sarcomas. J Transl Med. 2011; 9:200-200.
99. Gonzalez E, McGraw TE. The Akt kinases: Isoform specificity in metabolism and cancer. Cell Cycle. 2009; 8:2502-2508.
100. Fortier AM, Asselin E, Cadrin M. Functional specificity of Akt isoforms in cancer progression. Biomol Concepts. 2011; 2:1-11.

(Received February 27, 2017; Revised May 23, 2017; Accepted May 25, 2017)