[Frontiers in Bioscience E3, 968-977, June 1, 2011]

C-Myb function in the vessel wall

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1. ABSTRACT

C-Myb is a DNA-binding transcription factor that functions in apoptosis, proliferation and differentiation. The role of c-Myb in vascular injury has been investigated previously both *in vitro* and *in vivo,* where knock-down of c-Myb is known to lead to a reduction in proliferation and an increase in apoptosis of vascular smooth muscle cells (VSMCs). Reduction of c-Myb activity has also been shown to decrease neointimal formation *in vivo,* by reducing VSMC proliferation. In contrast, over-expression of c-Myb *in vivo* leads to increased survival rates in certain cell types. This review will look mainly at studies investigating c-Myb function in the vasculature, and evidence of signalling interactions which may be considered with regard to c-Myb as a possible target in the treatment of vasculoproliferative diseases.

2. INTRODUCTION

The proto-oncogene *c-myb* was originally isolated by Hall and colleagues (1) and is a transforming gene of two avian acute leukaemia viruses, AMV and E26 (2). The Myb gene family encode nuclear proteins that function in transcriptional transactivation. C-*myb* is the most widely studied and characterised (1) of the Myb family members, and functions in cell cycle progression, proliferation, differentiation and apoptosis (3). It is highly conserved throughout evolution and is present in all vertebrate and some invertebrate species (4).

The c-Myb protein consists of a DNA binding domain, transactivation domain and a negative regulatory domain (1, 3) (Figure 1). The major translational product of the c-*myb* proto-oncogene is a 75kD nuclear protein

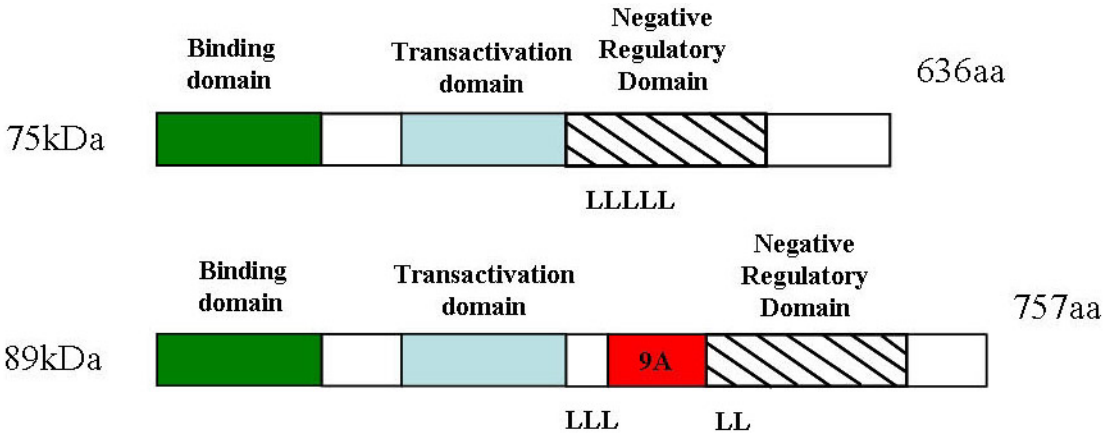

Figure 1. Structure of c-Myb gene family products. The DNA binding motif is unique and highly conserved. It makes up the first one third of the c-Myb molecule and is made up of three tandem 50 amino acid direct repeats - R1, R2 and R3. R2 and R3 are required for complex formation of Myb with DNA, while R1 is thought to be required for stabilisation of the complex. The domain also consists of regular tryptophan residues that form hydrophobic scaffolds, thought to maintain helix-turn-helix motifs contained within the R2R3 fragments. Arganine and lysine residues flanking the final tryptophan residue are thought to be critical for DNA binding activity of the c-Myb protein. Together, the DNA binding domain and the tryptophan repeats uniquely characterise the structure of the Myb family proteins. In the 89kDa protein (p89), exon 9A represents an additional 363 base pairs between exons 9 and 10. In the negative regulatory domain (NRD), a short sequence within the leucine zipper (L) known as the FAETL motif has been shown to be critical for transformation and transactivation of c-Myb. Image adapted from (1).

consisting of 636 amino acids, expressed in most haematopoietic tissues. In addition, an 89kD translational product is found to be expressed in avian, murine and human haematopoietic cells (1, 3).

C-Myb is involved directly in cell proliferation and cell cycle control; however its precise role remains unclear. Maximal activity of c-Myb occurs during late G_1 and S phase of the cell cycle (3), and c-Myb has also been shown to contribute to G_2/M cell cycle transition in human haematopoietic cells (5). Movement of calcium across the plasma membrane is observed in proliferating cells (6). C-Myb is a regulator of cytoplasmic Ca^{2+} pools in VSMCs, shown using dominant negative c-Myb (which lacks critical elements of the DNA binding domain) or antisense-c-*myb* (AS-ODN-c-*myb*; 'switches off' gene expression by complementary mRNA base pairing) *in vitro*; and furthermore this is independent of the stage of cell cycle progression (7, 8). C-Myb has also been shown to be a mediator of G_1/S – associated transcriptional repression of the Plasma Membrane Ca^{2+} ATPase pump in rodent VSMCs (7). More recent work by the same group concluded that c-Myb-dependent Ca^{2+} regulation functions through the IP3 receptor-1 promoter, where c-Myb has 17 putative binding sites (9).

In recent years, studies have been performed in several animal models to investigate the exact physiological roles of c-Myb with respect to the vascular system and also the signalling pathways it participates in. This review will look at the information gathered from these studies to date, and the possibilities for future work that may help to elucidate the functions of c-Myb in greater detail.

3. C-MYB AND HAEMATOPOIESIS

Perhaps the most widely used example of the role of c-Myb in cell differentiation is its involvement in haematopoiesis (1), and a considerable amount of research on c-Myb has focused on this area. Expression of c-Myb is higher in immature haematopoietic cells than terminally differentiated cells, suggesting that a general down-regulation takes place during differentiation (10). A number of studies have investigated the consequences of c-*myb* deletion/knock-down in haematopoietic tissues (11). It has been shown that c-Myb is critical for cell differentiation, yet development remains unaffected by the absence of c-Myb. Homozygous c-*myb* mutant mice die *in utero* on day 15 of gestation due to disruption of adult erythropoiesis, despite exhibiting normal vascular development and morphology (12), suggesting an important physiological role for c-Myb *in vivo*. Another study by Emambokus *et al.* which used a knock-down allele to express only 5-10% of normal wild-type c-*myb* levels resulted in the same number of haematopoietic progenitors as seen with wild-type levels *in vitro*, yet commitment and differentiation occurred earlier in mutants (13). In cells of haematopoietic origin, c-*myb* deficiency can lead to a decrease in platelets, T cells and B cells (11), which has the potential to be beneficial in altering the balance of inflammatory cells in the context of injury or vasculoproliferative diseases such as atherosclerosis. A significant amount of literature implicates T cells and B cells in such diseases (14). Furthermore, platelets are thought to play a role in the initiation of atherogenesis, due to expression of a wide range of cell surface molecules that are important in the immune response, such as ICAM-2, CD40 ligand, integrins and chemokines (15).

Table 1. Previous studies using c-Myb knockdown in the vasculature

Mode of Inhibition	Model	Observations	Reference
AS-ODN (pluronic gel)	Carotid balloon injury (rat)	Inhibition of VSMC proliferation	26
AS-ODN (EVAc matrix)	Carotid balloon injury (rat)	Inhibition of cell growth by 56.9% *in vitro*	27
Ribozyme - cationic lipid complex	*In vitro* (rat/porcine/human)	Inhibition of VSMC proliferation	29
AS-ODN (transport catheter)	Coronary angioplasty (porcine)	79% reduction in intimal-medial cross section	23
AS-ODN (hydrogel catheter)	Peripheral angioplasty (porcine)	VSMC proliferation reduced by 18%	28
AS-ODN (pluronic gel)	Interposition vein graft (rabbit)	38% reduction in mean intimal thickness of treated vein graft	25
MybEN transfection	*In vitro* (rat, rabbit & human)	Inhibition of VSMC proliferation/induction of apoptosis	32
AS-ODN	Coronary angioplasty (porcine) & *in vitro*	Enhanced VSMC and medial cell apoptosis following angioplasty	20
VSMC-specific MybEN expression	Carotid wire injury (mouse)	Reduced VSMC proliferation, neointimal formation, medial hyperplasia & remodelling	34
MybEN (fused with VP22 HSV)	*In vitro* (porcine)	Enhanced growth inhibition and apoptotic effects of MybEN-VP22 fusion	33
C-Myb$^{-/-}$ embryonic stem cells	*In vitro*	SM-markers decreased, contractility decreased	21

Examples of *in vitro* and *in vivo* studies investigating the effects of c-*myb* inhibition on the vasculature and/or vascular cells. These data clearly show that different methods of inhibition lead to similar effects, those being an inhibition of proliferation and stimulation of apoptosis. AS-ODN: antisense oligonucleotide; VSMC: vascular smooth muscle cell; MybEn: MybEngrailed.

4. *IN VIVO* EFFECTS OF C-MYB DEFICIENCY

Studies of c-*myb* deficiency in other cell types also show a functional role for c-Myb. Cre-mediated c-*myb* deletion in the brain (*c-myb-nestin-cre*) results in enlarged ventricular spaces, thought to be due to a disruption to neurogenesis (16). In colonic mucosa, c-Myb levels were shown to decrease upon cell differentiation and apoptosis, accompanied by a decrease in bcl-2, a principal mediator of apoptosis (17). In a transplant model, colon and small intestinal tissue from *c-myb*$^{-/-}$ mice that was placed under the kidney capsule of recipient adults failed to develop properly, and exhibited severe epithelial disorganisation, whilst the small intestine was unaffected (18). C-Myb expression has been reported in bovine (19) and porcine (20) VSMCs, in addition to ApoE$^{-/-}$ murine aortic VSMCs (Farrell and Holt, unpublished observations), and has been implicated in differentiation of contractile VSMCs, where c-*myb*$^{-/-}$ embryonic stem cell-derived embryoid bodies showed an absence of VSMC-like contractility and a decrease in VSMC-specific markers (21). This suggests that loss of c-Myb *in vivo* could have serious implications in relation to changing VSMC phenotype.

5. THE ROLE OF C-MYB IN VASCULAR PATHOPHYSIOLOGY

Due to the observations that c-Myb plays a role in cell cycle progression (7, 9, 19, 22, 23), it has been postulated that c-Myb could be a possible target for treating pathological VSMC proliferation (22). C-Myb has been targeted both *in vitro* and *in vivo* in an attempt to control cellular proliferation in injured vessels (Table 1). Characterisation of c-Myb mRNA in quiescent, serum-stimulated bovine VSMC cultures resulted in very low levels of c-Myb after two hours, whilst levels increased significantly by 18 hours (corresponding to the G(1)/S interphase), upon serum addition (19). In contrast, growth arrest occurred following treatment with AS-ODN-c-*myb,* preventing entry into the S phase of the cell cycle (19). Maximal c-*myb* levels have also been observed at 18 hours *in vivo* following injury with balloon angioplasty in porcine coronary arteries (23, 24). A study by our own group (20) using AS-ODN-c-*myb* treatment provided further information about cell-specific c-Myb expression (Figure 2), where maximum levels were noted in inflammatory cells of porcine coronary arteries using antisense oligonucleotides after 18 hours, and in VSMCs 3-7 days post-angioplasty (20). Anti-proliferative effects following c-Myb inhibition have been noted in other cell types including bone marrow-derived mononuclear cells and myeloid cell lines (1).

Other studies using AS-ODN-c-*myb* have been shown to inhibit proliferation of porcine VSMCs *in vitro* in a dose-dependent manner and reduce neointima formation *in vivo* following porcine percutaneous transluminal coronary angioplasty (PTCA), where intimal-medial cross sectional area was reduced by 79% compared with saline (Figure 3) (23). AS-ODN-c-*myb* delivered via gel-coated vein grafts in a rabbit model showed a 38% reduction in mean intimal thickness of vein grafts (25). Delivery of AS-ODN-c-*myb* via a pluronic gel to injured rat carotid arteries has been shown to reduce accumulation of intimal VSMCs (26), and furthermore reduced cell growth by 56.9% in rat VSMCs following carotid balloon injury (27). In a porcine angioplasty model, AS-ODN-c-*myb* delivery via a hydrogel catheter led to an 18% reduction in VSMC proliferation (28). Reduction in VSMC proliferation has also been observed *in vitro* following inhibition of c-Myb activity through the use of ribozymes (29). In relation to apoptosis, AS-ODN-c-*myb* treatment doubled apoptosis rates in VSMCs of angioplastied porcine coronary arteries *in vivo* compared to vessels injured by PTCA alone (Figure 3) (20). Treatment with AS-ODN-c-*myb in vitro* also increased apoptosis levels in VSMCs but no effect was observed in endothelial cells (20), suggesting that c-Myb may elicit cell-specific effects. Such evidence highlights the potential of c-Myb as a therapeutic target.

Further involvement of c-Myb interactions with apoptotic genes have been reported, including work performed by this group, where porcine VSMCs treated with AS-ODN-*c-myb in vitro* led to a decrease in levels of anti-apoptotic Bcl-x_L and a rise in pro-apoptotic Bad. In addition, activation of caspase-3 and -9 were observed following inhibition of c-*myb* using both AS-ODN-c-*myb* and the transactivational repressor Myb Engrailed (MybEn); however, no activation of caspase-8 was noted (Withers *et al.,* unpublished observations). These data

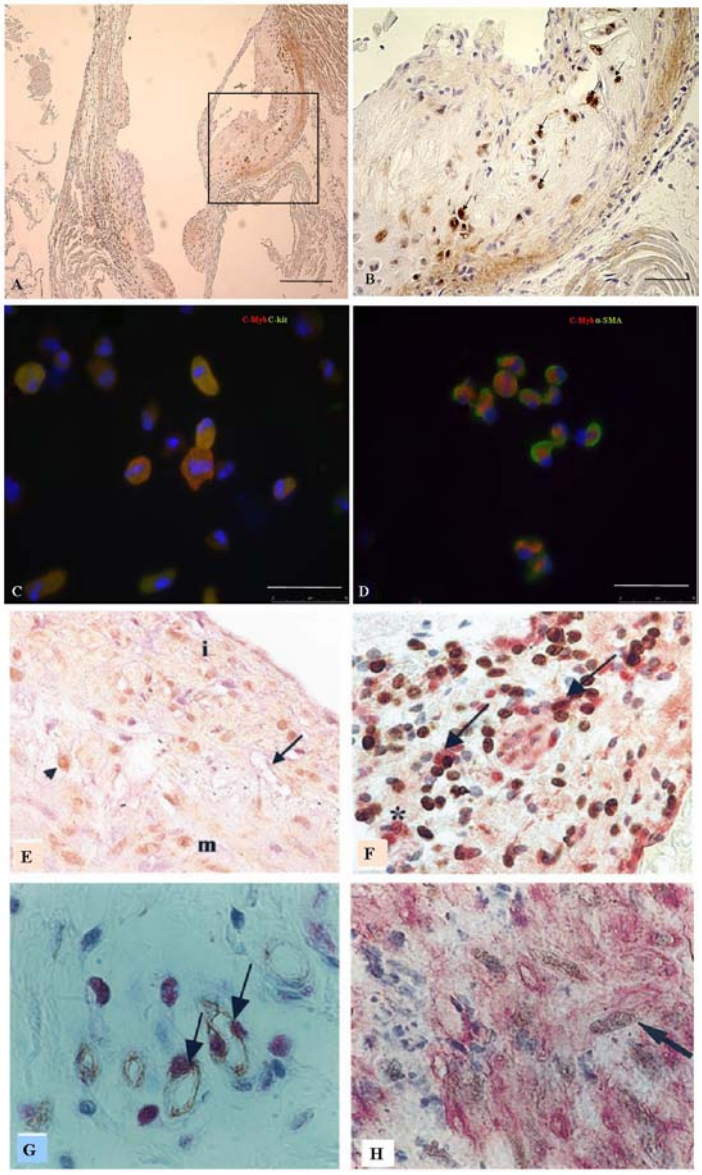

Figure 2. Expression of c-Myb in vascular disease and injury. (A) Immunolocalisation of c-Myb (brown) in atherosclerotic plaques of the aortic root. (B) C-Myb was localised in smooth muscle-like cells in the tunica media (arrows) and in unidentified cells scattered throughout atherosclerotic plaques (B, arrowheads). (C) C-kit (green) was present in isolated aortic cells and presented a similar pattern of localisation to that seen in c-Myb^{+} cells (red). A very small number of cells appeared to be sca-1+, and only a few in this group were sca-1^{+}c-Myb^{+} (data not shown). (D) Subsequent double labelling was carried out using a marker for α-SMA (green) in parallel with c-Myb (red), where the vast majority of cells stained were c-Myb^{+} α-SMA^{+}. (E) C-Myb^{+} cells in the media (m) and intima (i) of a porcine coronary artery 7 days post-angioplasty, x100. (F) Co-localisation of c-Myb (red) with inflammatory (CD68^{+}) cells (brown) in porcine coronary 6 hours post-angioplasty, x100. (G) C-Myb (red) co-localises with endothelial cells (brown) of adventitial microvessels in porcine coronaries 3 days post-angioplasty, x100. (H) C-Myb (brown) was found to co-localise with porcine coronary medial VSMC (red) 18 hours post-angioplasty. Image A, scale bar = 20 μm; B, 5μm; C-D, 50μm. Images E-H reproduced with permission from (20).

suggest that the inhibition of c-Myb leads to intrinsic or mitochondrial apoptosis.

In some cases, controversy exists regarding targeting of c-Myb through the use of antisense oligonucleotides. Anti-proliferative results using antisense oligonucleotides against *c-myb* have been suggested as non-specific (30). Additional vessel injury may result from the method used to deliver the therapeutic agent *in vivo,* as is seen with other vascular interventional treatments including angioplasty. Despite this, specific effects of antisense oligonucleotides have been thoroughly validated

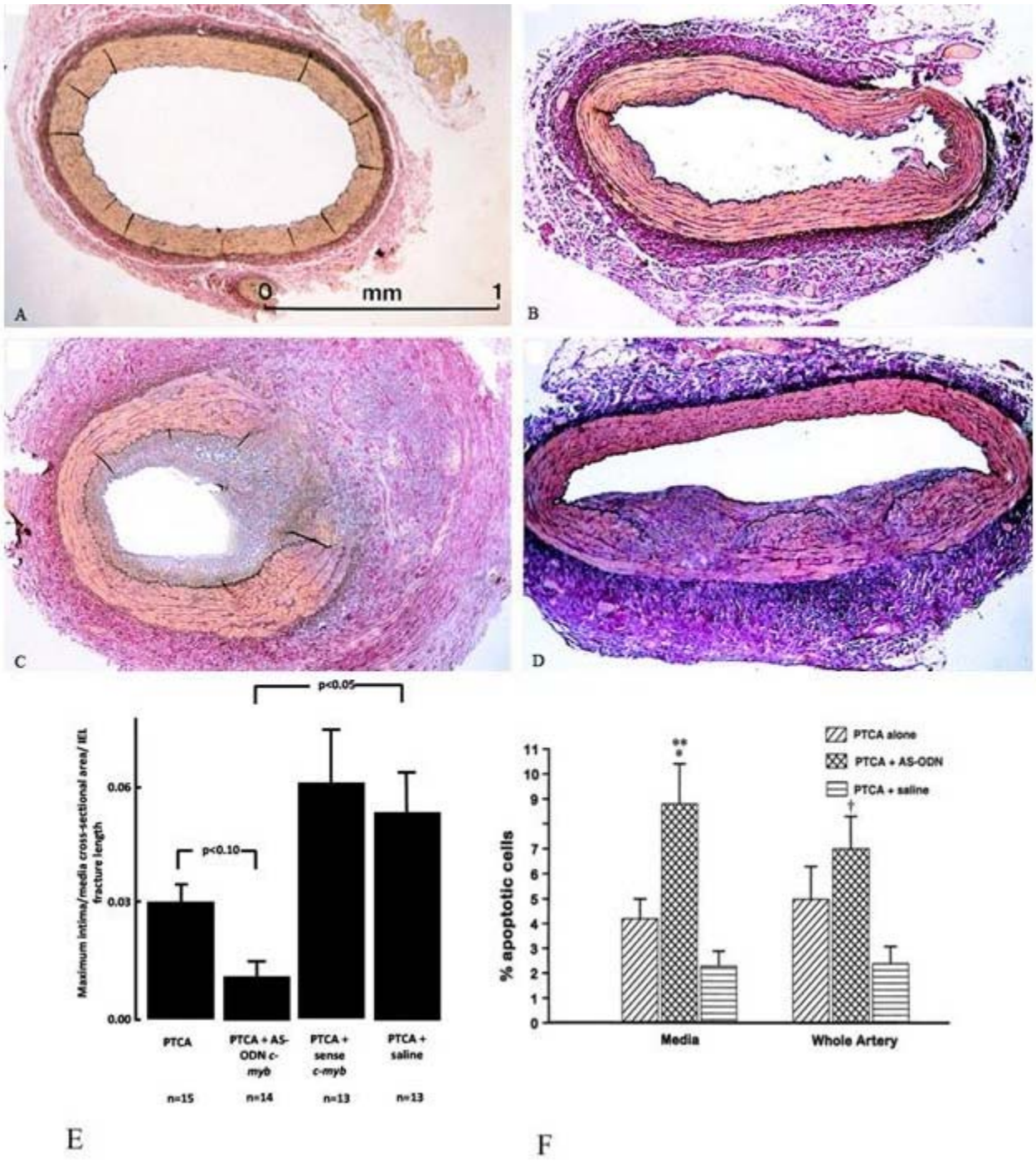

Figure 3. Effects of c-*myb* inhibition on VSMC proliferation and apoptosis *in vivo*. (A) A normal porcine coronary artery. (B) Artery harvested immediately after balloon PTCA, showing breached intima, internal elastic lamina (IEL) and media. (C) Artery harvested 4 weeks post-angioplasty. There are obvious breaches of the IEL, a thick neointima, and an adventitial reaction. (D) Artery 4 weeks post-angioplasty following local delivery of AS-ODN–c-*myb*. Despite two breaches of the IEL, neointima formation appears modest. (E) Measurements of intimal-medial cross section of porcine coronary arteries showed a significant reduction in those where AS-ODN-c-*myb* was delivered at time of angioplasty compared to sense (82% reduction), saline (79%) and angioplasty alone (63%). (F) C-*myb* inhibition by AS-ODN significantly increased apoptosis in media and whole artery of porcine coronaries when compared to saline or angioplasty alone, six hours post-angioplasty. Images A and B reproduced with permission from (23), and image C from (20).

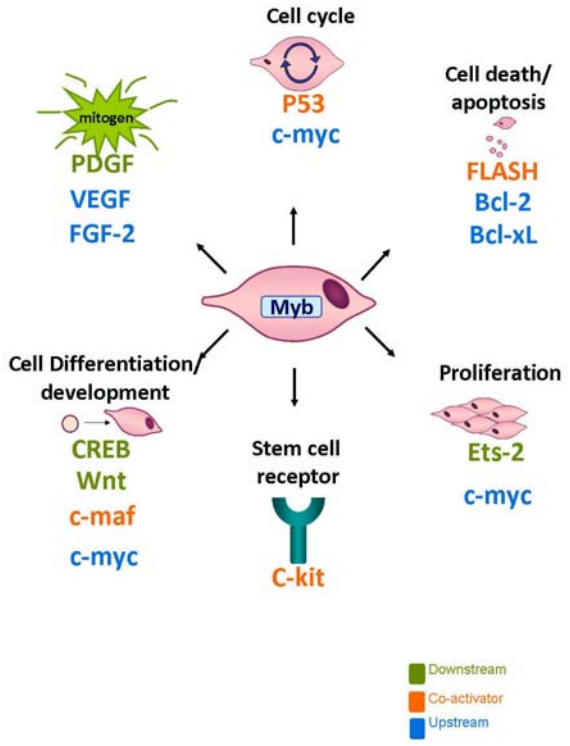

Figure 4. Evidence of c-Myb signalling interactions in the vasculature. Examples of some documented genes that are involved in signalling interactions with c-Myb, either downstream (green), upstream (blue), or as co-activators (orange). Certain genes have been documented as having direct effects on c-Myb-expressing cells, such as VSMCs, in the vasculature – via various mechanisms such as apoptosis (bcl-2, FLASH), proliferation (c-myc, Ets-2) or cell cycle progression (P53).

both *in vitro* and *in vivo* (23), and AS-ODN-*c-myb* treatments have been successfully used clinically in the treatment of chronic myelogenous leukaemia (31).

An alternative approach to antisense utilises Myb Engrailed (MybEn), a dominant negative fusion between the c-Myb DNA binding domain and the transcriptional repressor domain of the *Drosophila* Engrailed protein. This has been used in several studies investigating c-Myb function (32-34), and operates through repression of c-Myb transactivation (35). In a mouse model of carotid artery injury, MybEn was conditionally expressed in arterial VSMCs under control of the SM22α promoter (34). This resulted in a significant decrease in VSMC proliferation, neointima formation, medial hyperplasia and arterial remodelling, whilst the vasculature continued to exhibit normal morphology and function (34). An earlier study using MybEn constructs transfected into rat, rabbit and human VSMCs showed a reduction in VSMC proliferation *in vitro* in addition to induction of apoptosis (32). Fusion of MybEn with VP22, a herpes simplex virus-1 protein, further enhanced apoptotic effects of c-Myb in porcine VSMCs, and enhanced inhibitory effects on VSMC growth (33). Inhibition of proliferation by the use of conditionally active MybEn constructs has also been documented in T cells *in vitro* (24). Studies using dominant negative c-Myb in mouse embryonic fibroblasts which do not normally express c-Myb in abundance (and therefore are not dependent on c-Myb expression), resulted in G1 cell cycle arrest similar to that observed in c-Myb-expressing cells (36). This suggests that cell cycle progression is c-Myb dependent, even in cell populations that do not possess abundant levels of the protein.

Although the majority of evidence relating to c-Myb function in the vasculature is related to injury, particularly following surgical procedures, c-Myb expression has been noted in atherosclerotic plaques and the VSMC-rich medial layer of ApoE$^{-/-}$ aortae at both early and later stages of atherosclerosis (Farrell and Holt, unpublished observations). Interestingly, it has been noted in the same study that c-Myb expression levels are decreased in response to atherosclerotic plaque development in ApoE$^{-/-}$ mice, compared to wild-type levels, suggesting that c-Myb could be down-regulated as a result of an unknown atherogenic factor. It is not known whether c-Myb has a direct effect on plaque progression or stability during atherogenesis; yet these observations implicate c-Myb in another vascular pathology which is unrelated to interventional procedures.

It is clear from the evidence presented here that the balance between apoptosis and proliferation levels, if sufficiently controlled, may help in the treatment of vascular pathologies involving excessive VSMC proliferation including restenosis and neointimal hyperplasia, in addition to atherosclerosis. What is not apparent, however, is the effect of c-*myb* knock-down or ablation on downstream intracellular processes.

6. C-MYB SIGNALLING INTERACTIONS

Transcriptional regulation of c-Myb and information about its key target genes have yet to be fully elucidated. It has been established that c-Myb can be regulated by transcription factors including CREB binding protein which functions in terminal differentiation, and Ets-2 which has been previously implicated in cell proliferation (Figure 4) (1). Evidence suggests that c-Myb could be a downstream target of platelet derived growth factor (PDGF), where c-Myb rescued human aortic VSMCs from apoptosis that was induced through adenoviral infection with a truncated PDGF receptor (37). Additionally, c-Myb is phosphorylated and degraded indirectly by Wnt-1, an important developmental signalling protein (38). This occurs via TAK1 and subsequent binding of HIPK2 and NLK with c-Myb, and is thought to induce G1 arrest through inhibition of c-Myb-dependent activation of the c-myc promoter (38).

C-Myb has been implicated in regulation of fibroblast growth factor-2 (FGF-2), which is an important VSMC mitogen (39). A recent study has shown that c-Myb has the ability to activate the VEGF promoter in a murine myeloid cell line *in vitro*, yet such activation did not occur

due to DNA binding (40). It was suggested that the activation possibly occurred through interaction of c-Myb with another transcription factor bound to DNA sequences within the promoter (40). Similar observations about DNA-independent transactivation have also been made in studies involving c-Myb interactions with HSP70 (38) and bcl-2 (41). Research has shown that the anti-apoptotic gene bcl-2 is a target of c-Myb (41). A cytotoxic T cell line susceptible to apoptosis was protected in the presence of constitutive c-Myb, possibly as a result of induction of bcl-2 expression (41). Similarly, over-expression of both a degradation-resistant c-Myb mutant and bcl-2 in growth factor-deprived haematopoietic cells resulted in enhanced clonogenic potential and proliferative capacity (42). More recently, it has been documented in a conditional deletion mouse model that c-Myb promotes survival of thymocytes through upregulation of Bcl-x_L (43). *In vitro* studies using Jurkat and HL60 cells in ChIP assays and siRNA-mediated knockdown of c-*myb* have shown that c-myc, MAT2A and ADA are definitive targets of c-Myb (44). An earlier study, using tamoxifen-inducible MybEn expression in murine T cells *in vivo,* followed by subtraction screening showed a total of 29 targets regulated by Myb family members, particularly c- and b-Myb (45). These targets range from genes which are involved in the cell cycle (Mad111) and cell death (bcl-2, casp6 and cd53), as well as those important in cell adhesion and cytoskeletal regulation (Actinin-1).

Vital transcriptional co-activators of c-Myb include CBP/P300 (46) and p53 (47, 48). C-Myb also forms a complex with the transcription factor c-maf which in turn disrupts interactions of c-Myb with bcl-2, leading to reduced bcl-2 expression and apoptosis (49). FLASH (FLICE-associated huge protein), a component of the Fas-caspase-8 pathway is known to be a co-activator of c-Myb, and together they are found to associate with the MYC promoter in addition to ADA, another c-Myb target gene (50).

In relation to haematopoietic precursors, c-Myb is essential for expression of the c-kit receptor in erythroid cells (51) and in addition, c-Myb/c-kit interactions have been noted in neural crest development (52). C-Myb and c-kit co-expression has also been observed in $ApoE^{-/-}$ murine VSMCs (Farrell and Holt, unpublished observations). C-*myb* knock-down *in vivo* has recently been shown to alter the differentiation profile of haematopoietic stem cells (53). These authors have also shown that c-*myb* knock-down can alter monocyte and neutrophil number, where monocytes increase, and neutrophils decrease (53). This may have implications in inflammatory processes *in vivo* - if haematopoietic stem cell differentiation is affected, any beneficial contribution from those cells could ultimately be disrupted.

7. SUMMARY

From recent work that has been performed, evidence suggests that c-Myb may function in a variety of vascular signalling pathways. Targeted c-*myb* ablation *in vivo* could possibly lead to a reduction in important downstream transcriptional targets, such as the apoptotic factors Bcl-2 and Bim. There does not yet seem to be a definitive list of c-Myb target genes available for reference (44). It is clear that c-Myb has the potential to be a therapeutic target for a number of vascular disorders due to its warranted effects on apoptosis and proliferation.

The investigations into c-Myb, its signalling pathways as well as its roles in both apoptosis and proliferation (20, 23, 26, 34) implicate c-Myb as an important molecule in pathophysiology, both in and beyond the vasculature. The interaction of c-Myb with a wide number of signalling molecules serves to highlight that the effects of c-Myb are far-reaching, yet there is not a definitive list of c-Myb target genes available to reference. A significant amount of research is required in order to further our knowledge about this potentially exciting molecule in order to fully explore its potential future benefits.

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Abbreviations: VSMCs: vascular smooth muscle cells; ApoE: apolipoprotein E; AS-ODN: antisense oligonucleotide; PTCA: percutaneous transluminal coronary angioplasty; MybEn: Myb Engrailed; PDGF: platelet derived growth factor; FGF-2: fibroblast growth factor-2; FLASH: FLICE-associated huge protein.

Key words: c-Myb, apoptosis, proliferation, atherosclerosis, angioplasty, neointima, smooth muscle, knock-down, antisense, Myb Engrailed, Review

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