



The BD2 domain of BRD4 is a determinant in EndoMT and vein graft neointima formation

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ABSTRACT

Background: Vein-graft bypass is commonly performed to overcome atherosclerosis but is limited by high failure rates, principally due to neointimal wall thickening. Recent studies reveal that endothelial-mesenchymal transition (EndoMT) is critical for vein-graft neointima formation. BETs are a family of Bromo/ExtraTerminal domains-containing epigenetic reader proteins (BRD2, BRD3, BRD4). They bind acetylated histones through their unique tandem bromodomains (BD1, BD2), facilitating transcriptional complex formation and cell-state transitions. The role for BETs, including individual BRDs and their unique BDs, is not well understood in EndoMT and neointimal formation.

Methods and results: Repression of BRD4 expression abrogated TGFβ1-induced EndoMT, with greater effects than BRD2 or BRD3 knockdown. An inhibitor selective for BD2 in all BETs, but not that for BD1, blocked EndoMT. Moreover, expression of a dominant-negative BRD4-specific BD2 fully abolished EndoMT. Concordantly, BRD4 knockdown repressed TGFβ1-stimulated increase of ZEB1 protein – a transcription factor integral in EndoMT. *In vivo*, lentiviral gene transfer of either BRD4 shRNA or dominant negative BRD4-specific BD2 mitigated neointimal development in rat jugular veins grafted to carotid arteries.

Conclusions: Our data reveal the BD2 domain of BRD4 as a determinant driving EndoMT *in vitro* and neointimal formation *in vivo*. These findings provide new insight into BET biology, while offering prospects of specific BET domain targeting as an approach to limiting neointima and extending vein graft patency.

1. Introduction

Endothelial-mesenchymal transition (EndoMT) is a process involving endothelial cell (EC) switching to a mesenchymal-like cell state. EndoMT is now implicated in a growing number of disease conditions [1]. In addition to atherogenesis [2], EndoMT was recently identified as crucial for the failure of vein grafts [3], the bypass conduit surgical treatment of atherosclerosis in coronary and peripheral arteries. Despite their extensive use, vein graft failure is common, reaching 40% within 12–18 months after grafting [3]. Thus, a compelling rationale exists for understanding the regulation of EndoMT [1], which might allow for novel therapeutic options in an area with limited if any progress.

In response to specific extracellular stimuli, including transforming

growth factor (TGFβ1) exposure, ECs undergo a cell state transition, losing EC markers while manifesting proteins like smooth muscle actin (αSMA), calponin, and collagen-I that characterize smooth muscle cells (SMCs) [3]. Subsequent execution of EndoMT involves distal specific transcription factors, including ZEB1 (Zinc Finger E-Box Binding Homeobox), Snail, and Twist [1]. This EndoMT transcriptional reprogramming manifests as acquired non-EC phenotypes, including collagen production and cell migration [1]. EndoMT remains poorly understood, in contrast to epithelial-mesenchymal transition (EMT). While EndoMT and EMT overlap in some mesenchymal-like signaling pathways and cellular characteristics [1], distinct EndoMT mechanisms are increasingly being recognized [2,3]. Despite this progress, there has been limited insight into how context-dependent epigenetic regulations

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influence EndoMT programs [1].

The Bromodomain and ExtraTerminal domain-containing epigenetic reader proteins (BETs) have been recently identified as powerful determinants of differentiation and cell-state transitions [4,5]. BETs are comprised of family members BRD2, BRD3, BRD4, and BRD-t (testis-specific) [5], each containing two bromodomains (BD1 and BD2) that bind to acetylated lysines as marked on histones or present in transcription factors. Depending on cell types and environmental cues, BETs facilitate assembly of specific transcription factors with core transcription machinery to activate (or repress) a select set of genes that orchestrate cellular responses, including cell state transitions [6]. As we and others have shown, BET inhibition can block cell state transitions involving EC inflammation, cardiomyocyte hypertrophy and SMC proliferation [4,7,8]. Nevertheless, key questions remain regarding basic BET biology in vascular cells, including the relative contributions among different BET family members as well as the specific role of BD1 vs BD2 in BET-regulated responses. Specifically, BET involvement in neointimal formation in vein graft failure remains unexplored. Deeper understanding of BET biology may prove essential to targeting BETs for therapeutic purposes as is underway in oncology as well as heart disease [9,10].

Using selective genetic and pharmacological tools applied to *in vitro* EndoMT studies as well as an *in vivo* model of vein graft neointimal formation, we present data here dissecting out the specific roles of individual BET family members and their bromodomains in EndoMT and neointimal formation. Our data indicate that BRD4 plays a predominant role in EndoMT over BRD2 or BRD3, effects that depend on BD2 not BD1. Further mechanistic studies reveal that BRD4 controls TGF β 1-stimulated increases in ZEB1. *In vivo*, manipulation of BRD4 or BRD4-specific BD2 in the rat vein graft model supports an essential role for this epigenetic reader protein in neointimal formation. Taken together, these studies reveal details of BET biology that determine EC functional responses, BET involvement in EndoMT underlying cell state/identity transitions, and novel potential strategies for the unmet need of preventing vein graft failure.

2. Methods

2.1. Rat aortic EC culture, induction of EndoMT, and pretreatment with BET inhibitors

Rat primary aortic endothelial cells (ECs) were purchased from Cell Biologics (Cat.RA-6052). For cell expansion, the culture was maintained at 37 °C/5% CO₂ in the Complete Rat Endothelial Cell Medium containing growth factor supplement and 2% FBS (Cell Biologics, Cat. M1266); Accutase (Lifetechnologies, Carlsbad, CA) was used for cell detachment. The cells used for experiments were at passages 5–7. For induction of EndoMT, cells were starved in the Endothelial Cell Culture Basal Medium (Cell Biologics, Cat. M1266b) with 1%FBS for 6 h, and TGF β 1 (human recombinant, R&D Systems Inc., MN) was added to a final concentration of 50 ng/ml and incubated for 72 h or 96 h before cell harvest for mRNA or protein determination respectively. In the experiments using BET inhibitors, cells were pretreated with JQ1, RVX208 (Apexbio, Houston, TX, USA), or Olinone (Cat. GLXC-05021, Glix Laboratories, Southborough, MA, USA) at indicated concentrations, or vehicle control (equal volume of DMSO, Sigma-Aldrich, St. Louis, MO) for 2 h prior to induction of EndoMT with TGF β 1.

2.2. Quantitative real-time PCR (qRT-PCR) to determine mRNA levels

The assay method was described in our previous report [7]. Briefly, total RNA was isolated from cultured cells using the Trizol reagent (Invitrogen, Carlsbad, CA). Potential contaminating genomic DNA was removed by using gDNA Eliminator columns provided in the kit. RNA was quantified with a Nanodrop NP-1000 spectrometer, and RNA of 1 μ g was used for the first-strand cDNA synthesis (Applied Biosystems,

Carlsbad, CA). Quantitative RT-PCR was then performed using Quant Studio 3 (Applied Biosystems, Carlsbad, CA). The house keeping gene GAPDH was used for normalization. Each cDNA template was amplified in triplicate PerfeCTa SYBR® Green SuperMix (Quantabio) with gene specific primers, as listed in Table S1.

2.3. *In vitro* knockdown of BET family members with siRNAs

ECs were grown to 70% confluency in 6-well plates in the Complete Rat Endothelial Cell Medium with growth factor supplement. The BRD2-, BRD3-, or BRD4-specific siRNA was added to transfect ECs for overnight using the RNAi Max reagent (Thermo Fisher, cat.13778150). Cells were then recovered in the complete medium for 24 h. If EndoMT was induced, the culture was changed to the basal medium containing 1% FBS and incubated for 6 h prior to TGF β 1 stimulation. The siRNAs were ordered from Thermo Fisher (sequences listed in Table S2).

2.4. Adeno- and lenti-viral vectors for *in vitro* and *ex vivo* transduction

Adenovirus stocks for HA-tagged BRD4 (AdBRD4-HA, cat# 073066A) and empty vector control (AdNull, cat# 000047A) were purchased from Applied Biological Materials (Canada). An MOI of 100 was used for *in vitro* infection of primary rat aortic ECs. Cells were grown to 70% confluency in 6-well plates in the Complete Rat Endothelial Cell Medium with growth factor supplement, and adenovirus was added and incubated for 6 h followed by 24 h recovery in the complete medium. Cells post infection were incubated in the basal medium (1% FBS) for 6 h and then treated with TGF β 1 for 96 h before harvest for Western blotting analysis.

The piLenti-shRNA-GFP vectors for the production of a scrambled shRNA or an shRNA specific for rat BRD2, BRD3, or BRD4 were purchased from Applied Biological Materials (Canada). The most efficient sequences (of siRNAs, final products) are as follows: BRD2, CCACAAT GGC TTCTGTACCA; BRD3, AGTGAGTGTAT GCAGGACTTCAACAC CAT; BRD4, GCGAATCTAGCTCCTCTGACAGTGAAGAC. The lenti-viruses were packaged in Lenti-X 293 cells (Clontech, cat#632180) using a three-plasmid expression system (piLenti-siRNA-GFP, psPAX2 and pMD2.G) as we recently reported [7].

The lentiviral construct for the expression of GFP fusion (Fig. S1) with BRD2 or BRD4 or BD2(BRD4) was generated using a GFP-expressing lenti-vector (empty vector, control) that was kindly provided by Dr. Ming-Liang Chu (Guizhou Renmin Hospital, China). Molecular cloning and lentivirus packaging in Lenti-X 293 cells were performed following our previously reported method [7]. Briefly, before harvesting viruses, a minimum of 5 \times 10⁵ infection unit (IFU)/ml in supernatant was guaranteed using Lenti-x GoStix (Takara, cat.631243). The crude viral solution was then concentrated using Lenti-x concentrator (Takara, cat.631232) to a final concentration of 108–109 IFU/ml using the Lenti-x qRT-PCR Titration Kit (Takara, cat.631235). MOI was determined based on the fluorescence from GFP expression following the transduction with lentiviral vectors. An optimal MOI of 10 was used for *in vitro* infection of primary rat aortic ECs with lentivirus to express GFP alone (empty vector), BRD2-GFP, BRD4-GFP, or BD2(BRD4)-GFP. With this MOI we could achieve ~70–80% infection rates without noticeable cytotoxic or cytostatic effects; a higher MOI could result in EC death. Cells were grown to 70% confluency in 6-well plates in the Complete Rat Endothelial Cell Medium with growth factor supplement (Cell Biologics, Cat. M1266), and lentivirus plus polybrene (Santa Cruz., Cat.sc-134,220) was added and incubated for 6 h followed by 24 h recovery in the complete medium. Cells post infection were incubated in the basal medium (1% FBS) for 6 h and then treated with TGF β 1 for 96 h before harvest for Western blotting analysis. The use of lentiviruses for *in vivo* experiments is described below.

2.5. Western blotting to determine changes of protein levels

Cells were collected and lysed in RIPA buffer (Thermo Fisher, cat.89900) on ice, and a protease inhibitor cocktail (Thermo Fisher, cat.87785) was included. Cell lysates were quantified for protein concentrations using the Bio-Rad DC™ Protein Assay kit (cat.5000112) and loaded to a 10% SDS-PAGE gel. The protein transfer to a PVDF membrane and incubations with primary and secondary antibodies were performed following our established methods [7]. The antibody sources and dilution ratios are listed in Table S3. Specific protein bands on the blots were illuminated by applying enhanced chemiluminescence reagents (Pierce), recorded by an Azure C600 Imager (Azure Biosystems), and quantified using NIH Image J. The intact uncropped blot images can be found in Figs. S2–S7.

2.6. Ex vivo gene transfer and rat jugular vein graft model

All animal studies conform to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and protocols approved by the Institutional Animal Care and Use Committee at The Ohio State University. Male Sprague-Dawley rats (Charles River; 300–330 g) were used.

The model of rat autologous external jugular vein to common carotid artery reversed graft interposition was performed with modifications based on a method previously described [11]. Briefly, prior to surgery, rats were anesthetized with isoflurane (5% for inducing and 2.5% for maintaining anesthesia). After a midline incision, the posterior facial branch of the external jugular vein was exposed by aseptic incision in the ventral neck. An 8 mm segment of the vein was harvested and temporarily placed in cold Hank's Balanced Salt Solution (HBSS, containing Ca^{2+} and Mg^{2+}) until use for *ex vivo* gene transfer.

For knockdown of BRD2, BRD3, or BRD4, or for overexpression of BD2(BRD4)-GFP in vein grafts, *ex vivo* lentiviral transduction was performed [3]. Briefly, prior to grafting, jugular vein segments (explants) were incubated for 4 h (37 °C/5% CO_2) with the lentivirus expressing BRD2 (or 3 or 4)-specific shRNA or BD2(BRD4)-GFP in the RPMI 1640 medium containing viral particles (> 107 IFU/ml) and heparin (10 U/ml).

For vein grafting, the right common carotid artery was dissected and severed between two ligatures generating two ends. Each end of the carotid artery was clamped together with an overwrapped plastic cuff and the ligature was removed. Each end of the carotid artery was then everted over the cuff and secured with a suture, and a vein explant (after viral transduction) was sleeved over both everted arteries on cuffs and secured with sutures.

To prevent thrombosis, prior to vein graft interposition, heparin was injected (subcutaneous, 300 U/kg body weight), and the lumens of carotid arteries and vein grafts were periodically flushed with heparinized saline during the grafting procedure. Clamps were then removed to restore blood flow, and skin wounds were closed using clips. Following subcutaneous injection of heparin (200 U/kg), animals were recovered and returned to husbandry. Rats were euthanized at 4 weeks post vein grafting, and the grafts were collected for histological analysis.

2.7. Morphometric analysis of intimal hyperplasia (IH)

At 4 weeks after the grafting surgery, the whole vein graft except the perianastomotic regions was harvested and immersed in 4% paraformaldehyde for 24 h and then paraffin-embedded. Paraffin sections (5 μm thick) were excised from the grafts at equally spaced intervals and then H&E-stained for morphometric analysis. Planimetric parameters as follows were measured on the sections and calculated using Image J as described in our previous report [7]: area inside external elastic lamina (EEL area), area inside internal elastic lamina (IEL area), lumen area, intima area (= IEL area - lumen area), and media area

(= EEL area - IEL area). Intimal hyperplasia was quantified as a ratio of intima area versus media area (I/M). Measurements were performed by a student blinded to the experimental conditions. There were two animal groups each including 5–6 rats: one group grafted with a vein that was transduced with lentivirus to express scrambled shRNA (or GFP) control, and the other group with a vein transduced with lentivirus to express BRD4 shRNA (or BRD4-specific BD2). The data from all 4–6 sections were pooled to generate the mean for each animal. The means from all the animals in each treatment group were then averaged, and the standard error of the mean (SEM) was calculated.

2.8. Statistical analysis

To confirm one result, at least three independent repeat experiments were performed. Data are presented as mean \pm SEM. Two-condition comparison was analyzed with Student's *t*-test using Prism version 4.0 (GraphPad Software). Multi-condition comparison was analyzed with one-way ANOVA followed by Bonferroni *post hoc* test. Significance was set to $P < .05$.

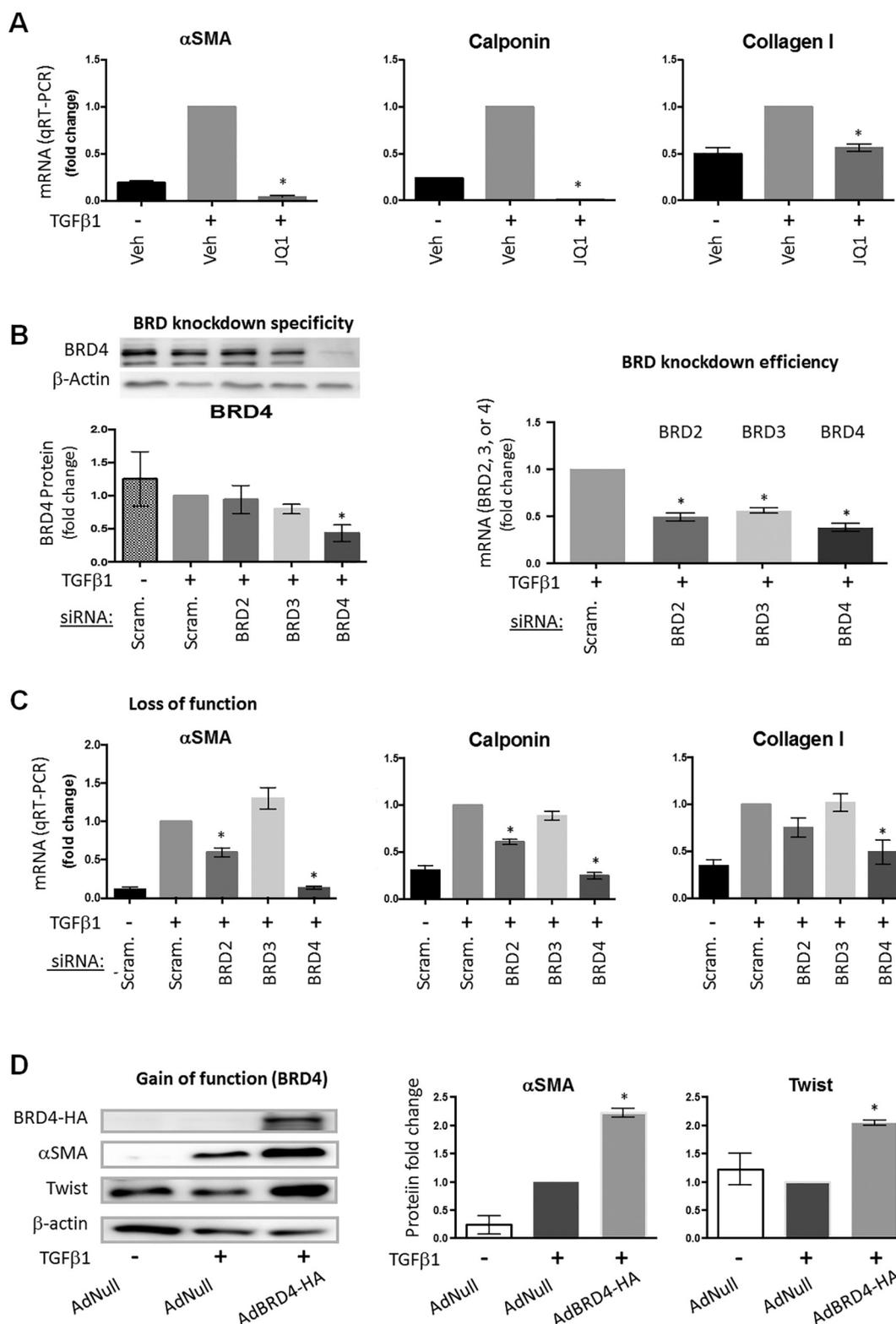
3. Results

3.1. Treatment with pan-BET bromodomain inhibitor JQ1 abolishes TGF β 1-induced EndoMT in rat primary aortic ECs

To investigate the BET family involvement in EndoMT, we studied rat primary aortic EC responses to TGF β 1 – a canonical inducer of EndoMT [1,3], doing so in the presence or absence of the pan-BET inhibitor JQ1 [12]. JQ1 reversibly binds both BD1 and BD2 in all BETs, thus disrupting the interaction between BRD2, 3 and 4 and acetylated lysines on histone tails and certain transcription factors [6]. EndoMT was monitored by detecting mRNA induction of three well-established EndoMT markers in ECs, namely αSMA , calponin, and collagen-I. Cells were pre-treated with vehicle or JQ1 (1 μM) prior to TGF β 1 stimulation. As expected, TGF β 1 induced EndoMT as indicated by a 2 to 4-fold induction of αSMA , calponin, and collagen-I mRNAs; in contrast, in the presence of JQ1, this cell state transition was completely abolished (Fig. 1A).

3.2. BRD4 plays a predominant role in TGF β 1-induced EndoMT

Given these potent effects of pan-BET inhibition on EndoMT, we next considered the relative contributions of BRD2, 3, or 4 in driving this EC response. As currently no BET member-specific inhibitors exist, we validated (Fig. 1B) and then employed siRNAs specific to each BRD in TGF β 1-induced EndoMT experiments (Fig. 1C). While BRD2 knockdown reduced TGF β 1-stimulated αSMA expression by ~30%, BRD4 silencing completely blocked the action of TGF β 1, reverting EC expression of αSMA mRNA to basal levels seen in unstimulated ECs (scrambled siRNA, no TGF β 1). BRD3 knockdown showed no effect on αSMA expression, slightly but not significantly increasing its mRNA over the TGF β 1-stimulated level. Similar patterns of BRD knockdown effects on calponin and collagen-I were observed: BRD2 siRNA reduced TGF β 1-stimulated expression by ~30%, BRD3 siRNA had no effect while BRD4 siRNA effectively reduced their mRNAs to basal, unstimulated levels (Fig. 1C). Further confirming the specific role of BRD4, its overexpression *via* adenovirus (Fig. 1D) or lentivirus (Fig. S1) in ECs significantly increased protein levels of αSMA , a canonical EndoMT marker, and Twist, a transcription factor involved in EndoMT [1,13]. Thus, while pharmacologic pan-BET inhibition blocks TGF β 1-induced EndoMT, specific genetic manipulation of individual family members indicates BRD4 as the dominant as well as sufficient BET determinant of EndoMT.



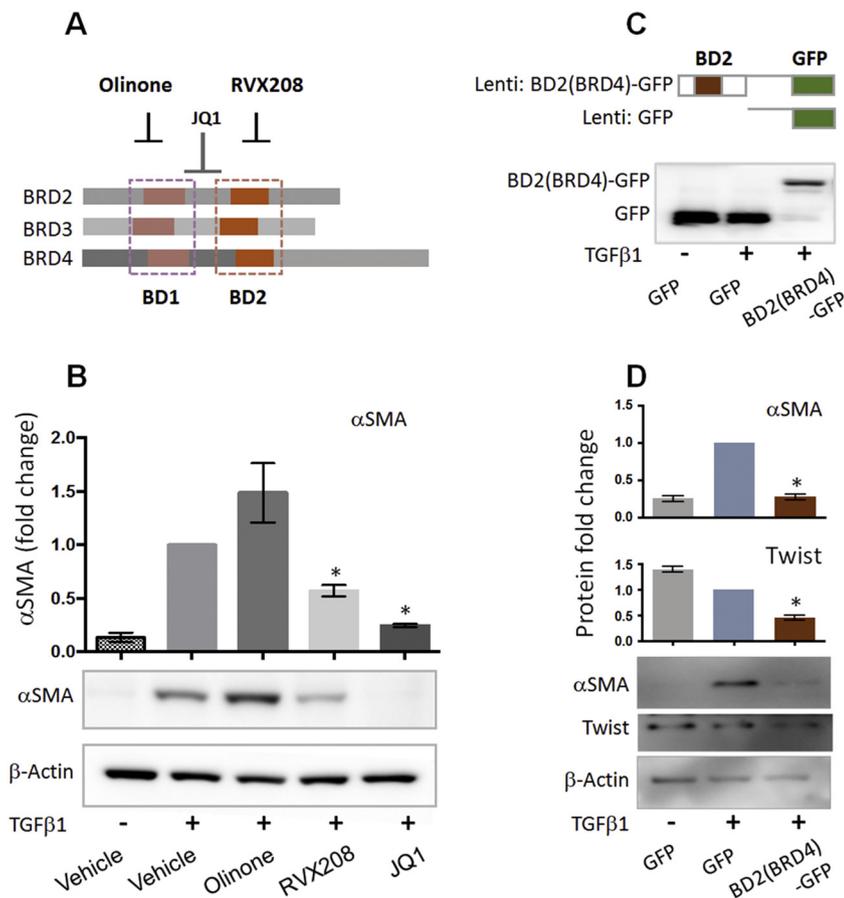
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3.3. Blocking BD2 but not BD1 of the BET family proteins abrogates TGFβ1-induced EndoMT

Since bromodomains within BETs determine their localized binding to specific acetyl modification of histone tails and subsequent transcriptional effects [5], we investigated whether the two highly homologous BET bromodomains [12] play differential roles in EndoMT.

Pharmacologic BET inhibitors exist that have been reported to have selective BD-blocking effects, namely RVX208 as inhibiting BD2 and Olinone as preferentially targeting BD1 (Fig. 2A), albeit without discrimination between BRD2, 3 and 4 [14,15]. We found that while pretreatment with RVX208 significantly decreased TGFβ1-stimulated αSMA protein levels as compared to vehicle control (+TGFβ1), an equivalent concentration of Olinone had no such effect (Fig. 2B). JQ1

Fig. 1. Either pan-BET inhibition or selective BRD4 silencing abrogates TGF β 1-induced EndoMT. **A.** The pan-BET inhibitor JQ1 represses TGF β 1-induced mRNAs of EndoMT markers. As described in detail in Methods, rat primary aortic ECs were starved for 6 h in the endothelial cell culture basal medium containing 1% FBS, and then pre-treated with 1 μ M JQ1 or vehicle control (equal amount of DMSO) for 2 h prior to adding TGF β 1 (final 50 ng/ml). Cells were harvested at 72 h after TGF β 1 stimulation and used for qRT-PCR assays. **B.** Efficiency and specificity of BET family member knockdown. ECs were transfected overnight with a scrambled siRNA or an siRNA specific for BRD2, BRD3, or BRD4, recovered in the complete medium for 24 h, and then starved in the basal medium for 6 h prior to adding TGF β 1. After 72 h and 96 h of TGF β 1 stimulation respectively, cells were harvested for qRT-PCR and Western blotting. **C.** siRNA targeting of BRD4 abolishes TGF β 1 induction of EndoMT. In the knockdown experiments (see B), the ECs stimulated (or not) with TGF β 1 for 72 h were used for qRT-PCR determination of mRNA levels of EndoMT markers. **D.** Gain-of-function: Adeno-viral expression of BRD4-HA. ECs were transduced for 6 h with AdNull (control) or AdBRD4-HA in the complete medium and recovered in fresh complete medium for 24 h. Cells were then starved for 6 h in the basal medium containing 1% FBS before TGF β 1 stimulation (96 h) followed by Western blotting. Quantification (A–D): For each independent repeat experiment, the control value (vehicle, scrambled siRNA, or AdNull, in the presence of TGF β 1) is presented as 1, and the values from other conditions are normalized to this control. Normalized values from independent repeat experiments (n = 3) are averaged (mean \pm SEM); *P < .05 compared to the normalizing control.



reverted TGF β 1-stimulated α SMA expression to the unstimulated basal level. A lesser RVX208 effect than JQ1 on α SMA expression was likely due to their different BD2-binding affinities (\sim 500 nM vs 50 nM, respectively) [15].

Given the predominant role of BRD4 in EndoMT (Fig. 1C) and the evidence for BD2 accounting for the bromodomain effect on EndoMT in all BETs (vs BD1, Fig. 2B), we investigated if the BD2 domain in BRD4 mainly accounted for BRD4's action in directing EndoMT. To address this, we utilized a lentiviral vector to express the BRD4-specific BD2 domain (Fig. 2C) that serves as a dominant-negative competitor to endogenous BD2, as previously established [16]. Indeed, compared to the empty vector (GFP control), expression of GFP-tagged BD2(BRD4) completely abolished TGF β 1 stimulation of α SMA protein expression (Fig. 2D) while also significantly reducing Twist expression. Together, these results support BD2 as determining the action of BRD4 in TGF β 1-induced EndoMT and suggest targeting BD2 in BRD4 would have the greatest impact on EndoMT.

3.4. BRD4 silencing averts TGF β 1-stimulated ZEB1 protein up-regulation

ZEB1 has been recently identified as a key transcription factor governing transcription programs for both EMT and EndoMT [1,17]. In addition, Snail and Twist have also been shown to be important for EMT, although their differential and/or cooperative functional relationships with ZEB1 remain to be understood [13]. Given BETs as an epigenetic mechanism for coupling transcription factors to gene expression programs and the data presented for BET regulation of EndoMT, we next investigated BETs involvement in controlling these novel transcriptional mediators of EndoMT. TGF β 1 increased ZEB1 protein, which was completely repressed in the presence of JQ1, reducing this protein to non-stimulated basal levels (Fig. 3A). We then used siRNAs to dissect the roles of specific BET family members. While BRD4 siRNA abolished TGF β 1-stimulated increases in ZEB1 protein, siRNA to either BRD2 or BRD3 had no significant effect on ZEB1 (Fig. 3B). Importantly, all three siRNAs had similar efficiency in repressing their respective BET member (Fig. 1B), thus eliminating variable knockdown as an explanation for these effects. Although TGF β 1

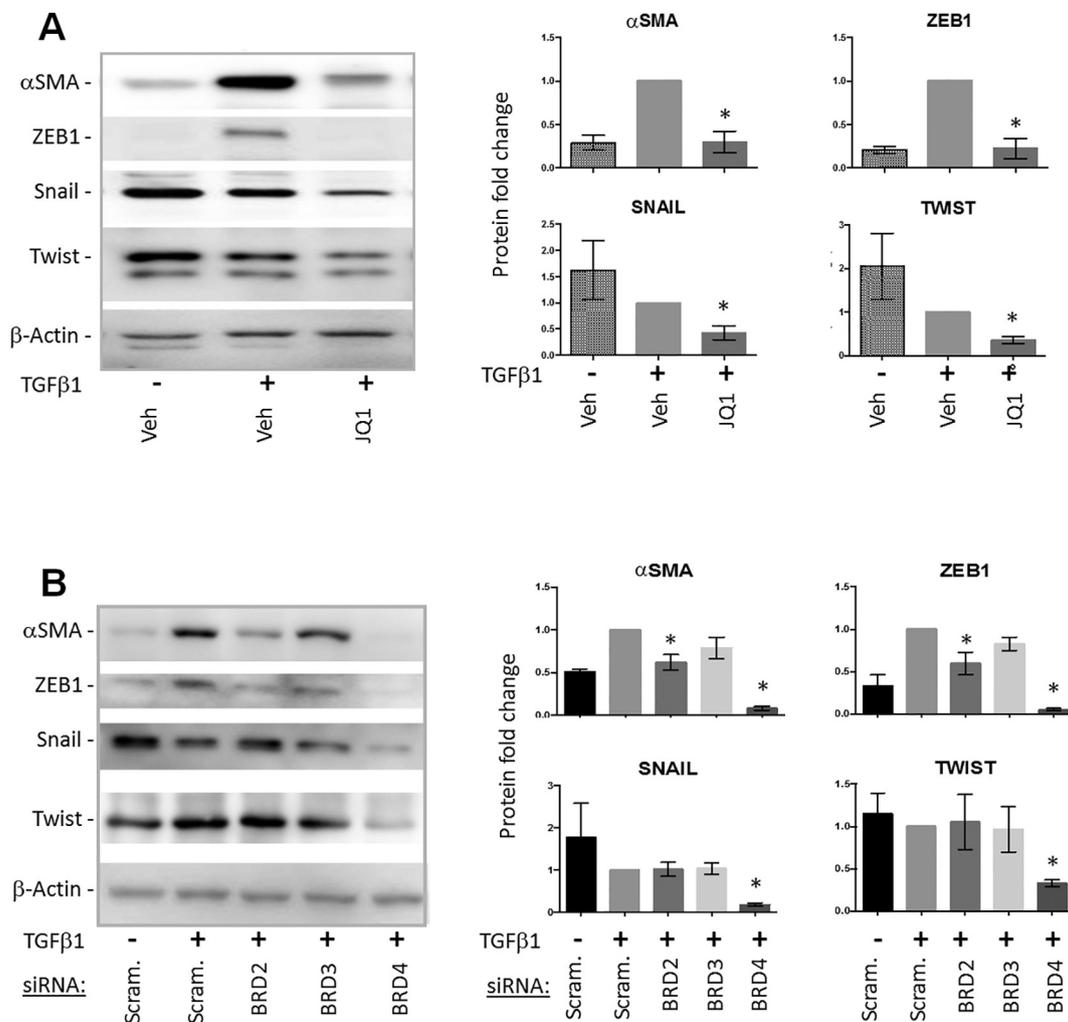


Fig. 3. BET inhibition blocks TGFβ1-induced increase of the EndoMT-associated master transcription factor ZEB1. **A.** Effect of pan-inhibition of BETs with JQ1. Experiments (JQ1 pre-treatment and TGFβ1 stimulation) were performed as described in Fig. 1A. At 96 h after TGFβ1 stimulation, ECs were harvested for Western blotting analysis of αSMA and EndoMT-associated transcription factors. **B.** Effect of knockdown with an siRNA for BRD2, BRD3, or BRD4. Experiments (siRNA transfection and TGFβ1 stimulation) were performed as described in Fig. 1C except that cells were harvested for Western blotting at 96 h after TGFβ1 stimulation. Quantification of Western blots was performed as described for Fig. 2; mean ± SEM; n = 4; *P < .05 compared to the normalizing control.

did not increase Snail and Twist protein levels, both JQ1 (Fig. 3A) and BRD4 siRNA (Fig. 3B) markedly reduced Snail and Twist protein levels whereas BRD2 and BRD3 siRNAs had no significant effects. These results indicate BETs, and more specifically, BRD4, as a mechanism controlling the expression of ZEB1, a central mediator in EndoMT.

3.5. Lentiviral transgene of BRD4 shRNA or dominant-negative BRD4-specific BD2 mitigates neointima formation in rat vein grafts

Given recent evidence for EndoMT as being critical to neointima formation [1,3], our data for BETs, and more specifically BRD4 regulation of mediators and markers of EndoMT suggested BRD4 manipulation would decrease neointima formation in vein grafts *in vivo*. To pursue this, we employed an established cuff technique [11] in a rat vein graft model to investigate specific BRD2, BRD3 and BRD4 involvement in neointimal thickening. This vein grafting model allowed for uptake of lentivirus expressing BRD4-specific shRNA or dominant-negative BRD4-specific BD2 domain by the intact endothelium of rat jugular vein explants (Fig. 4). The explants were incubated with lentivirus *ex vivo* for 4 h prior to their grafting onto carotid arteries (diagrammed in Fig. 4A). To assess the effectiveness of lentiviral infection to express BRD4-shRNA, we harvested and homogenized the infected vein grafts to determine BRD4 mRNA levels. The data showed reduced

BRD4 (and αSMA) mRNAs (Fig. 4B). Moreover, immuno-staining of GFP in the vein grafts infected with either the lentiviral vector of GFP control or BD2(BRD4)-GFP showed robust GFP expression, indicative of effective infection (Fig. 4C).

In order to determine the functional effect of *in vivo* transgenic manipulation of BRD4 or its BD2 domain, vein grafts were harvested 4 weeks after grafting and analyzed, including measurement of intima/media area ratio (I/M) as a measure of neointimal lesion formation [7]. Lentiviral transduction of either BRD2 or BRD3 shRNA in vein graft explants had no effect on I/M ratio as compared to empty lentiviral control vectors (Fig. S8). However, in striking contrast, lentiviral BRD4-shRNA transduction significantly reduced the I/M ratio by ~65% as compared to control vectors (Fig. 5, A and B). Furthermore, lentiviral expression of dominant-negative BD2(BRD4) also effectively mitigated neointima formation, as indicated by a 78% decrease of the I/M ratio compared to control (Fig. 5, C and D). These results provide *in vivo* evidence that BRD4, but not BRD2 or BRD3, and more specifically, the BD2 domain in BRD4, critically mediates neointima formation in the rat vein graft model, findings consistent with our *in vitro* data (Figs. 1–3).

Furthermore, in view of the well-documented role of BRD4 in promoting cell proliferation — a vascular cell behavior key to neointima formation [7], we immuno-stained vein graft sections for proliferating cell nuclear antigen (PCNA), an established marker of vascular cell

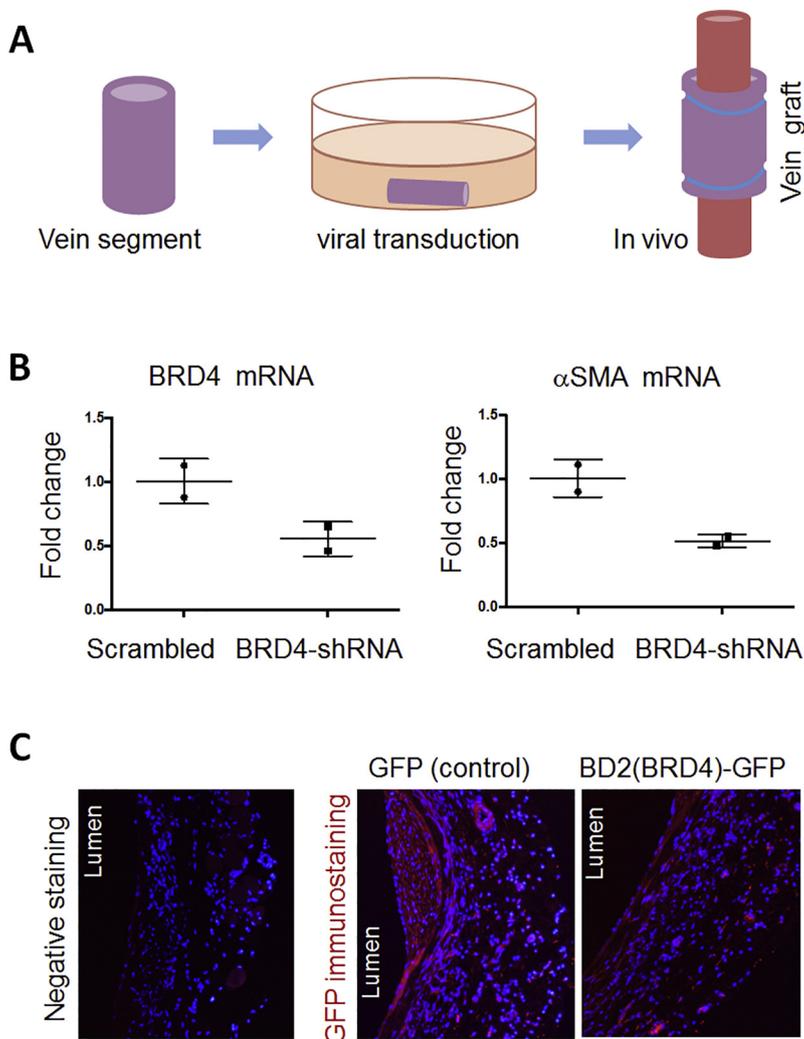


Fig. 4. *Ex vivo* lentiviral transduction of rat vein explants for *in vivo* transgene expression after grafting to arteries. *Ex vivo* lentiviral transduction of rat jugular vein segments and their grafting to carotid arteries are described in detail in Methods. A. Diagram of *ex vivo* lentiviral transduction of a rat jugular vein followed by grafting to a carotid artery. A cuff-assisted suturing (non-stitching) technique was applied. B. Effective *in vivo* knockdown of BRD4. Rat vein explants were transduced with lentivirus expressing BRD4-shRNA or scrambled shRNA (control). The vein grafts were collected 7 days after grafting and homogenated for qRT-PCR determination of BRD4 or α SMA mRNA levels. In each plot, each data point was obtained from one rat used in an independent experiment. C. GFP expression after lentiviral transduction. Vein explants were transduced with lentivirus expressing GFP (control) or dominant-negative BD2(BRD4)-GFP. The vein grafts were collected 4 weeks after grafting, and their cross-sections were immuno-stained (red) for GFP. Blue is DAPI staining. Negative staining was performed without the primary antibody. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proliferation. As shown in Fig. 6, vein graft transgenes of both BRD4-shRNA and dominant-negative BD2(BRD4)-GFP significantly reduced PCNA-positive cells compared to their respective controls. These results are consonant with our finding that the BD2 domain of BRD4 is a determinant of vein-graft neointima formation. However, without lineage tracing, we were not able to distinguish the sources of these proliferative cells, e.g. ECs or smooth muscle cells.

4. Discussion

We provide *in vitro* and *in vivo* evidence here for the BET bromodomain protein BRD4 as the specific BET family member that controls the EndoMT process, doing so by coordinating the endothelial transcriptional program induced by TGF β 1, including established EndoMT markers like α SMA as well as novel transcription factors like ZEB1. *In vitro*, BRD4 (vs BRD2 or BRD3) silencing is predominant and sufficient to block EndoMT, with this BRD4 effect dependent on BD2 but not BD1. *In vivo*, either BRD4 knockdown or dominant-negative BD2(BRD4) expression represses neointima formation, a key step in vein graft failure involving EndoMT.

Although an intervention commonly employed to relieve ischemia, vein grafting is a flawed strategy, with almost half of grafts failing in 4 years or less due primarily to flow-limiting neointima formation [3]. As a result, intense interest has focused on neointima formation and thus far unsuccessful attempts to modulate this vein graft response. Recent studies highlight EndoMT as a novel, fundamental driver of neointima formation in vein grafts as well as atherosclerosis [2,3].

During EndoMT, the endothelium undergoes transcriptional reprogramming and cell state transition, losing endothelial properties and expressing SMC-like markers. Although the identification of EndoMT has been a significant advance, thus far, no clear targets for therapeutically modifying EndoMT responses have emerged.

BETs have recently emerged as epigenetic regulators that control differentiation and cell state transitions, doing so by localizing master transcription factors and core transcriptional machinery at multiple discrete, marked chromatin sites through BET binding to acetylated lysines on histone tails. For various reasons, BET targeting is being pursued intently in oncology, including BET rearrangements as a direct cause of some cancers, BET involvement in defining specific gene cassettes underlying myeloma, and the development of specific BET inhibitors with favorable pharmacokinetic profiles [5,12]. More recent studies from us and others have strongly implicated BETs in cardiovascular responses involving cell state transitions, with BET inhibition blocking cardiac hypertrophy in response to trans-aortic constriction [8], the TNF α -induced NF κ B program in ECs and atherosclerosis [4], and SMC proliferation/migration [7], among others. Given these findings, we hypothesized BETs as potential novel determinants of the unique cell state transition/trans-differentiation found in EndoMT and subsequent neointima formation, as borne out here. While the common use of pan-BET inhibitors like JQ1 generally precludes elucidation of relative BRD2, BRD3 or BRD4 contributions to vascular responses, we establish here that EndoMT depends on BRD4, and more specifically, the BD2 of BRD4.

Consistent with a prominent effect of BET inhibition on blocking

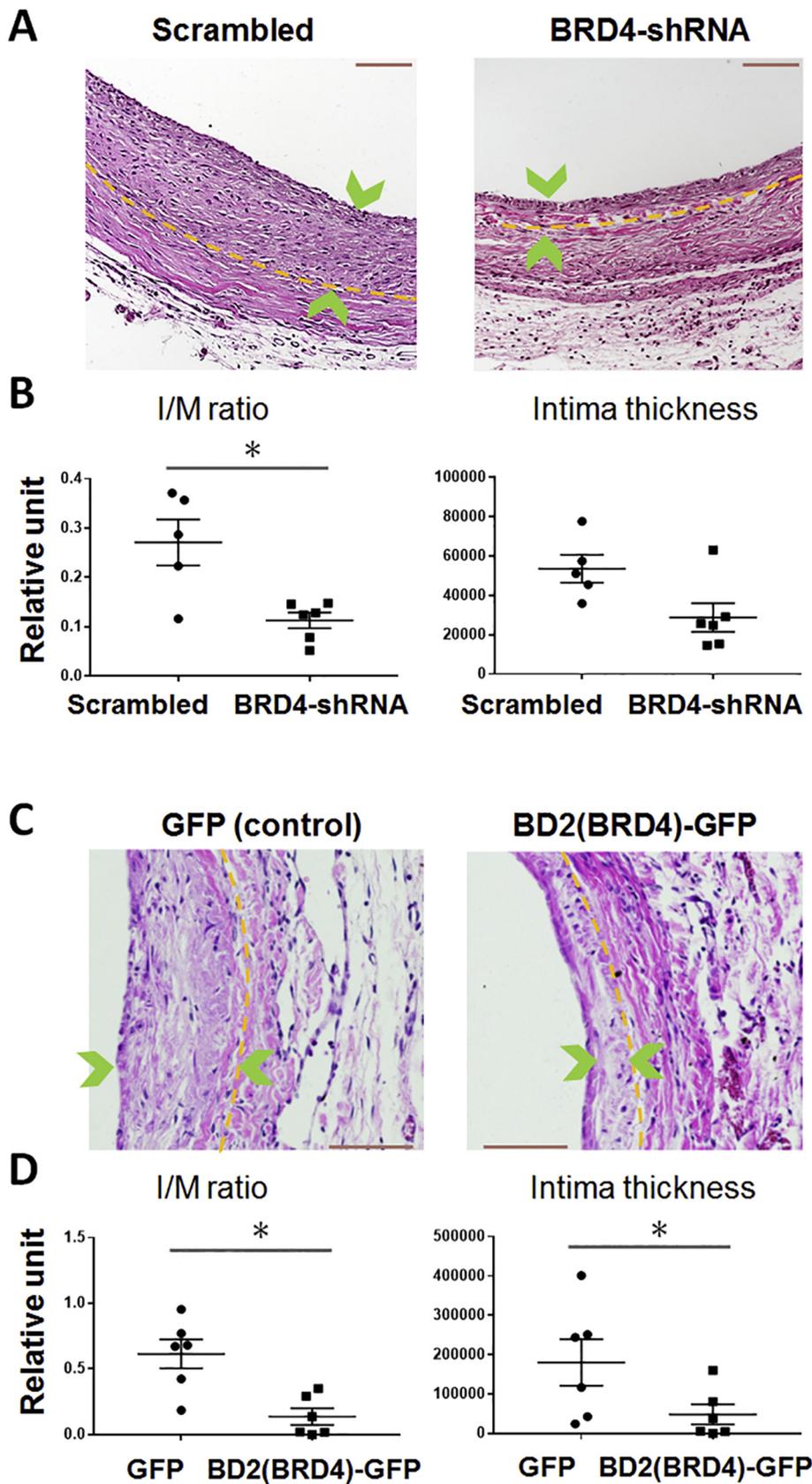


Fig. 5. Transduction of BRD4-shRNA or dominant-negative BD2(BRD4) lentivirus into vein grafts inhibits neointimal formation *in vivo*. *Ex vivo* lentiviral transduction and grafting of rat jugular veins were performed as in Fig. 4. Vein grafts were harvested 4 weeks after grafting; cross-sections were H&E-stained for analysis of the intima/media (I/M) area ratio. A and C. Representative H&E-stained cross-sections of vein grafts transduced with a lentiviral vector to express BRD4-shRNA (or scrambled control) or dominant-negative BD2(BRD4)-GFP (or GFP control), respectively. The thickness of neointima is indicated between a pair of green arrowheads; dashed line marks the internal elastic lamina (IEL). Scale bar: 100 μ m. B and D. Quantified morphometric data. Mean \pm SEM; n = 5–6 rats; *P < .05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

EndoMT observed herein, JQ1 has been reported to mitigate EMT in carcinomic [17,18] and pulmonary [19] epithelial cells, although without identification of the specific BET family members or BDs involved. Interestingly, a very recent report identified BRD2 as the key

regulator of EMT with BRD3 and BRD4 playing an opposite role [20]. This study, which contrasts with our finding of EndoMT being dependent on BRD4, may align with other studies identifying BRD4 action as varying as a function of cell-specific as well as stimulus-dependent

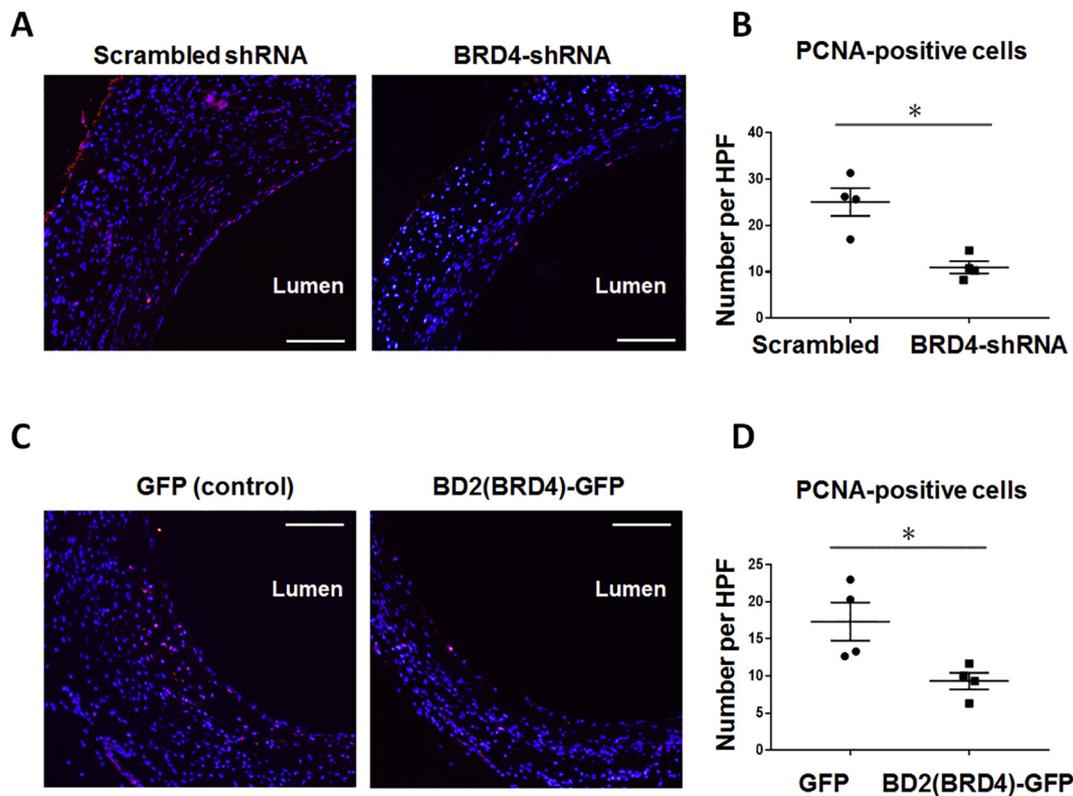


Fig. 6. Transduction of BRD4-shRNA or dominant-negative BD2(BRD4) lentivirus into vein grafts reduces proliferative cells. Experiments were performed as in Fig. 5. Cross-sections were immuno-stained for the proliferation marker PCNA. A and C. Representative PCNA-stained cross-sections of vein grafts transduced with a lentiviral vector to express BRD4-shRNA (or scrambled control) or dominant-negative BD2(BRD4)-GFP (or GFP control), respectively. Blue is DAPI staining. Scale bar: 100 μ m. B and D. Quantified data. Mean \pm SEM; n = 4 rats; *P < .05. HPF: high power field. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

contexts [6]. Examples include non-overlapping inflammatory gene regulation in macrophages [21] versus ECs [4], Myc regulation in myeloma [22] but not cardiomyocytes [8] and in stimulated T cells [23]. Alternatively, experimental issues may underlie observed differences in BET responses. While TGF β 1 was used to stimulate EndoMT here, no specific stimulus was used in investigating BET involvement in breast cancer EMT [20]. Another factor in dominant BRD4 versus BRD2 action may involve our studies being done in primary rat aortic ECs not immortalized cancer cell lines. Finally, EndoMT and EMT are overlapping yet distinct processes, with likely both shared as well as distinct regulatory mechanisms. In fact, during the submission of our manuscript [24,25], another report also showed an important role of BRD4 in the endothelial to mesenchymal transition in a disease context of cardiac fibrosis [26].

The potent role of BETs in controlling transcriptional programs in differentiation as well as pathologic states like cancer and atherosclerosis has prompted interest in better understanding BET action. Our data provides new insight into fundamental aspects of BET biology in vascular cells, with evidence that BET regulation of EndoMT depends on the second (BD2) but not the first (BD1) of the two tandem bromodomains present in BRD2, BRD3 and BRD4 [6]. BRD4 is unique in possessing a C-terminal domain, which has a reported critical role in co-activating transcription [5]. While BRD4 is recruited to specific gene loci via bromodomain binding to acetylated sites, the interaction of its C-terminal domain with the transcription elongation factor (pTEFb) facilitates RNA polymerase II activation. Whether the C-terminal domain present in BRD4 but absent in BRD2 [5], explains BRD4's more dominant role (vs BRD2) in directing the EndoMT program requires further study. Similarly, whether BD1 might be more important than BD2 in other cell types or settings will require additional investigation including use of approaches employed here. Despite their significant

sequence homology and structural similarity [12], our data highlights distinct effects mediated through BD2 versus BD1, variations that may have functional importance given the prospect of selective BD targeting for therapeutic purposes, as has been raised [5,14,15].

The EndoMT cell state transition can be viewed as a form of cellular reprogramming, with parallels to physiologic processes like differentiation and also pathologic issues like oncogenic transformation. Our identification of BRD4 as coordinating a TGF β 1-induced EndoMT transcriptional program provides new insight into the burgeoning interest in this mesenchymal transition. We show BRD4 regulation orchestrates changes in ZEB1, Snail, and Twist, downstream mediators of EndoMT [1]. Although not previously studied in terms of vascular biology, BRD4 has been shown to interact with Twist on chromatin in breast cancer cells [13]. We show TGF β 1-induced EndoMT increased ZEB1 protein but reduced Snail and Twist levels, with either the BET inhibitor JQ1 or BRD4 silencing abolishing these responses and preventing changes in EndoMT phenotypic markers like α SMA (Figs. 1 and 3). Of note, it has been recently identified that metabolic factors, such as endothelial fatty acid oxidation (FAO), regulate TGF β 1-induced EndoMT as well [27]. *In vivo*, earlier evidence indicated marked upregulation of TGF β 1 and its signaling pathway in human and animal vein grafts that developed neointima [28,29]. Importantly, a recent lineage-tracing study [3] provided compelling evidence for that through TGF β 1-activated EndoMT, endothelial-derived cells contributed significantly to the formation of vein-graft neointima in a grafting model very similar to ours. It is therefore conceivable that in our current study, BRD4, particularly its BD2 domain, played an important role in the neointimal pathogenesis probably as a determinant of EndoMT. However, as a limitation of this study, we could not distinguish the relative contribution of EndoMT from that of smooth muscle cell pathology. Both processes are driven by the BRD4 epigenetic activity, as

indicated by the data presented herein and in our previous study [7]. Nevertheless, our findings argue for further consideration of BET involvement in transcriptional regulation of EndoMT and its functional impact on the development of vessel-narrowing neointima in vein grafts.

5. Conclusions

By dissecting the functional roles of BET family members and BD domains, we reveal here that BRD4, and more specifically, the BRD4-specific BD2 but not BD1, plays the predominant role in TGF β 1-induced EndoMT, a key process in vein graft failure. As seen in other settings, we show BRD4 coordinates changes of transcription factors upon cell state transitions. These findings reveal novel mechanisms involved in EndoMT and BRD4 action, underscore selective BET family member action, and have implications for ongoing efforts at BET inhibition for therapeutic purposes, including support for selective BET bromodomain effects. Our results also suggest inhibition of BD2 in BRD4 as a possible strategy for the unmet need of preventing vein graft failure.

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Competing interests

The authors have declared that no competing interests exist.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cellsig.2019.05.005>.

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