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TP508 accelerates fracture repair by promoting cell growth over cell death

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Abstract

TP508 is a synthetic 23-amino acid peptide representing a receptor-binding domain of human thrombin. We have previously shown that a single injection of TP508 accelerates fracture healing in a rat femoral fracture model. To understand how TP508 acts at the protein level during fracture healing, we compared the translational profiles between saline-control and fractured femur at six time points after TP508 treatment using the second generation of BD Clontech™ Antibody Microarray. Here, we demonstrate that TP508 accelerates fracture healing by modulating expression levels of proteins primarily involved in the functional categories of cell cycle, cellular growth and proliferation, and cell death. The majority of those proteins are physically interrelated and functionally overlapped. The action of those proteins is highlighted by a central theme of promoting cell growth via balance of cell survival over cell death signals. This appears to occur through the stimulation of several bone healing pathways including cell cycle-G1/S checkpoint regulation, apoptosis, JAK/STAT, NF-κB, PDGF, PI3K/AKT, PTEN, and ERK/MAPK.

Keywords: Protein expression profile; Pathway analysis; Bone repair; Rat; Protein antibody array

TP508, a 23-amino acid peptide representing a thrombin-binding domain for a specific class of thrombin receptors, has a wide range of therapeutic effects on tissue repair [1]. Early studies showed that a single topical application of TP508 increased the breaking strength of wounds by approximately 80% over saline-controls and shifted the healing curve forward by approximately 4 days [2]. The same application also accelerated wound closure in normal rat skin [3] and surgically induced ischemia [4]. Significant effects of TP508 were also seen on hard tissue repair in fracture and distraction osteogenesis model systems. A single injection of TP508 into the fracture site increased bone strength by approximately 40% in 2-month-old rats [5] and accelerated repair of fresh fractures in 10-month-old rats [6], whereas three injections of TP508 administered in a rabbit distraction osteogenesis model led to earlier bone consolidation [7]. Similar therapeutic effects of TP508 on bone and cartilage repair models have also been observed with TP508 delivered in controlled release polylactoglycolide (PLGA) microspheres [8,9]. More recently, Fife and her colleagues have demonstrated that TP508 stimulates healing of human diabetic foot ulcers in a placebo-controlled phase 1/2 study [10].

The cellular mechanisms by which TP508 accelerates tissue repair involve early regulatory events in initiation and promotion of the cellular cascades underlying tissue repair. These events include early recruitment of inflammatory cells to the site of injury [6], induction of chemotaxis of

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human osteoblasts and microvascular endothelial cells [11], stimulating angiogenic sprouting to an extent similar to or greater than VEGF [12], increasing proliferation in less differentiated cells of the chondrocytic lineage, and stimulating matrix production at later stages of chondrocyte differentiation [13].

The molecular mechanisms of TP508 action in enhancing tissue repair have been a focus for recent studies. Wang and her colleagues [6] have shown that the increased fracture healing by TP508 is directly linked to the enhanced expression of a number of growth factors, inflammatory mediators, and angiogenesis-related genes, which altogether activate bone healing pathways favoring faster cell proliferation. More recently, Schwartz et al. found that TP508 is able to block chondrocyte apoptosis (personal communication). Given the fact that tissue healing is a coordinated action between cell growth and cell death pathways, these initial findings motivated us to hypothesize that TP508 accelerates tissue repair by promoting cellular growth over cell death pathways. To test this hypothesis, we used the BD Clontech™ Protein array to investigate translational changes between TP508-treated and saline-control samples in a rat femoral fracture model.

Materials and methods

Animals. Male Sprague–Dawley laboratory rats were obtained from Harlan (Indianapolis, IN) and housed at the research animal laboratory under conditions of 12 h light, 12 h darkness, ambient temperature of 20–23 °C, and relative humidity of 35–60%. Experimental animal procedures were in compliance with animal welfare regulation and approved by the OrthoLogic Research Department.

Experimental design and tissue collection. Ten-month-old male rats weighing from 400 to 500 g each were used in this study. Standard closed fractures of the right femur midshaft were created using the device and method as described by Bonnarens and Einhorn [14]. One hour after fracture, TP508 at doses of 0 (saline-control), 1, and 10 μg in 100 μl saline was percutaneously injected into the fracture site of each rat in each of the respective groups. Before tissue collection, the rats were euthanized by intraperitoneal injection of 2 ml Euthasol (Delmarva Labs, Midlothian, VA). One centimeter of fractured femurs were harvested at six time points (days 1, 4, 7, 14, 21, and 28) with three replicates (3 groups × 6 time points × 3 replicates). In addition, three intact, age-matched rat femurs (three replicates) were used as baseline controls. Fractured femurs were carefully dissected and cleaned to ensure no muscle contamination. The midshafts encompassing 1 cm of fractured femur were cut off using a sterile dremel saw blade and frozen in liquid nitrogen until protein extraction.

Protein labeling and antibody array hybridization. Protein extraction and labeling were performed using BD Clontech™ Protein Extraction & Labeling Kit as detailed previously [15]. In brief, 150 mg of bone samples were thawed and homogenized in non-denaturing buffer (Clontech), and then diluted after the protein concentration had been measured with the BCA kit (Pierce, Rockford, IL, USA). Each protein sample was labeled with Cy3 and Cy5 dye (Amersham) separately before being passed through a PD desalting column (Amersham). The Cy5- and Cy3-labeled proteins were then mixed and added to Antibody Microarray 500 array. The arrays were hybridized at room temperature for 30 min before a series of washes. The slide was dried and scanned using a GenePix 4000B scanner (Axon Instruments). The initial quantification of array images was performed using GenePix Pro 6.0 software. Signal intensity for each protein was determined by averaging background subtracted medium intensities from Cy3 and Cy5 channels.

Data analyses. Data normalization and comparison analyses were performed using GeneSpring 7.3.1 (Silicon Genetics). The data were normalized to median intensity (per chip) to allow relative comparisons between samples. The thresholds for selecting statistically differentially expressed proteins were set at a relative difference >1.2-fold and $p < 0.05$ (1-way ANOVA parametric test was performed assuming variances unequal). Cluster analysis was performed using D-Chip. The default clustering algorithm was used [16]. Global functional analyses, network analyses, and canonical pathway analyses were performed using Ingenuity Pathway Analysis 5.0 (Ingenuity® Systems, www.ingenuity.com). The statistic description for these analyses can be found on the Ingenuity website.

Results

TP508 significantly altered translational profiles at the early time points on days 1, 4, and 7 but had a limited effect on the later time points on days 14, 21 and 28 (Fig. 1). For the three early time points, the high dose was more effective than the low dose ($p < 0.05$). Since altered proteins substantially overlapped between low and high doses, the subsequent functional characterizations were based on the data derived from three early time points at high dose.

Compared to saline-control, 82, 142, and 153 proteins were differentially expressed on days 1, 4, and 7, respectively. The false discovery rates for these statistically changed proteins at the respective time points were 2.2%, 8.4%, and 4.6%. Although the differentially expressed proteins shared only 15 common proteins across three time points (Fig. 2A), they fell into similar functional categories. As shown in Fig. 2B, those proteins at three early time points were all significantly enriched by the functional groups of cell death, cell cycle, and, cellular growth and proliferation. The proteins from these three functional groups represent 54%, 58%, and 59% of all differentially expressed proteins on days 1, 4, and 7, respectively. We therefore closely examined these proteins and their temporal changes (Supplementary Figure 1A–C). The specific groups of proteins were associated with each time points for all three functional categories. In the cell cycle category, the dominant function of differentially expressed proteins experienced a

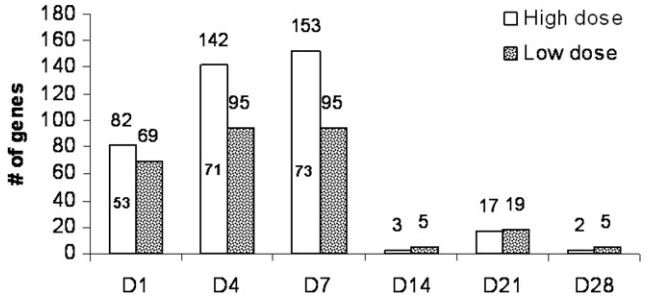

Fig. 1. The number of differentially expressed proteins induced by TP508 at different time points. TP508 altered more proteins on days 1, 4, and 7 than on days 14, 21, and 28, and had a greater effect at high dose than at low dose. The numbers on the top of bars represent the number of differentially expressed proteins. The numbers inside the open bars represent the number of common changes between the high dose and low dose.

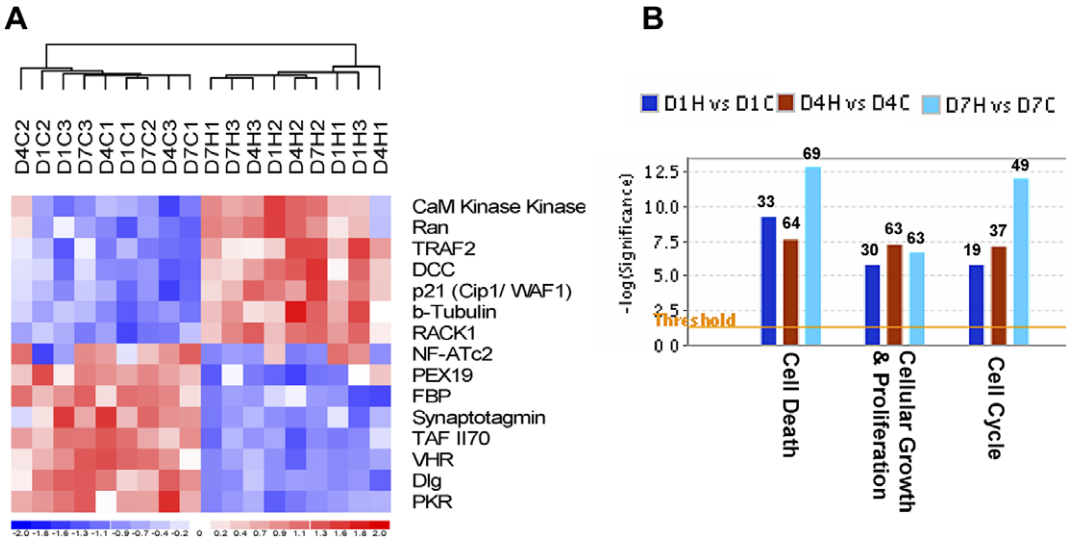

Fig. 2. Commonly changed proteins and significantly enriched functional categories across three early time points. (A) Cluster analysis of 15 commonly changed proteins. TP508-treated and non-treated samples fell into two distinctive groups. Seven proteins were stimulated (top) and eight were suppressed (bottom) by TP508. (B) Three significant functional groups across three time points. *Y* axis is $\log_2$ transformed significance (*p* value). $p = 0.05$ is equivalent to *y* value of 1.3 (horizontal line). Numbers on the bars represent the total number of differentially expressed proteins.

transition from regulating G2/M phase on day 1 ($p < 0.007$), regulating interphase on day 4 ($p < 0.002$) to regulating cell stage on day 7 ($p < 0.01$). Furthermore, those up-regulated proteins are primarily associated with the initiation of interphase, initiation and re-entry into cell stage, and cell cycle progression. In cellular growth and proliferation category, the most noteworthy observation was that 11 out of 14 kinases were up-regulated and 2 out of 2 phosphatases were down-regulated on day 4. This trend was not obvious on days 1 and 7. The up-regulated proteins are mainly involved in cell formation, quantity, growth, and proliferation. In the cell death category, several classic pro-apoptosis genes were up-regulated, including DIABLO, CASP14, CASP8, BIK, and BAK1 while anti-apoptosis gene, BIRC4, was down-regulated on day 4. Once again this trend was not extended to day 7. In contrast, many up-regulated proteins on day 7 promote cell survival, of which COX-2, NOS2A, Calcineurin, fibronectin, COPS5, VHL, and PIK3R1 have established functions in promoting bone formation by either stimulating osteoblasts or suppressing osteoclasts.

Fig. 3 shows a cluster analysis resulting from a list of differentially expressed proteins between fractured controls and TP508-treated samples on day 7. With all samples divided into two classes, all fractured control samples fell into one group while all treated samples and non-fractured control samples fell into another group. These two distinctive clusters indicate that TP508-treated profiles are more similar to the intact profiles compared to the fractured profiles.

Among the differentially expressed proteins (82 on day 1, 142 on day 4, and 153 on day 7), 62, 122, and 128 were eligible for network analysis (focus genes). Table 1 shows the first two networks, which represent 71% (day 1), 42% (day 4), and 60% (day 7) of the proteins eligible for network analysis. The analysis further revealed that the top functions represented by the first two networks are cell cycle (36 out of 49), cellular growth and proliferation (45 out of 63), cell death (55 out of 69), which are consistent with the most significant functional categories identified by functional analysis (Supplementary Figure 2). The data also demonstrate that those proteins are functionally or physically correlated.

Pathway analyses showed that differentially expressed proteins between the fractured control and TP508-treated femur were significantly enriched in 18 signaling pathways in at least two of the three early time points (Fig. 4). Of those, cell cycle-G1/S checkpoint regulation, PPAR, NF-κB, PDGF, VEGF, PI3K/AKT, IGH-I, SAPK/JUK, insulin receptor, and ERK/MAPK signaling pathways have been shown to be activated in the early stage of normal bone healing [15], while JAK/STAT, PTEN, EGF, apoptosis, calcium, GM-CSF, integrin, and nitric oxide signaling pathways were uniquely activated by TP508 treatment.

Discussion

The key finding of this study was that TP508 has a significant effect on promoting bone healing at early stage of repairing processes, corresponding to inflammation (day 1), intramembranous ossification (day 4), and chondrogenesis (day 7), whereas, it has a limited effect at later stage of healing, including endochondral ossification and remodeling. This could primarily attribute to the short half-life of TP508 (less than 12 h in the fracture site). A single injection 1 h post-fracture is unlikely to have a significant impact on the later processes like bone remodeling. However, tissue repair requires a tightly coordinated temporal expression

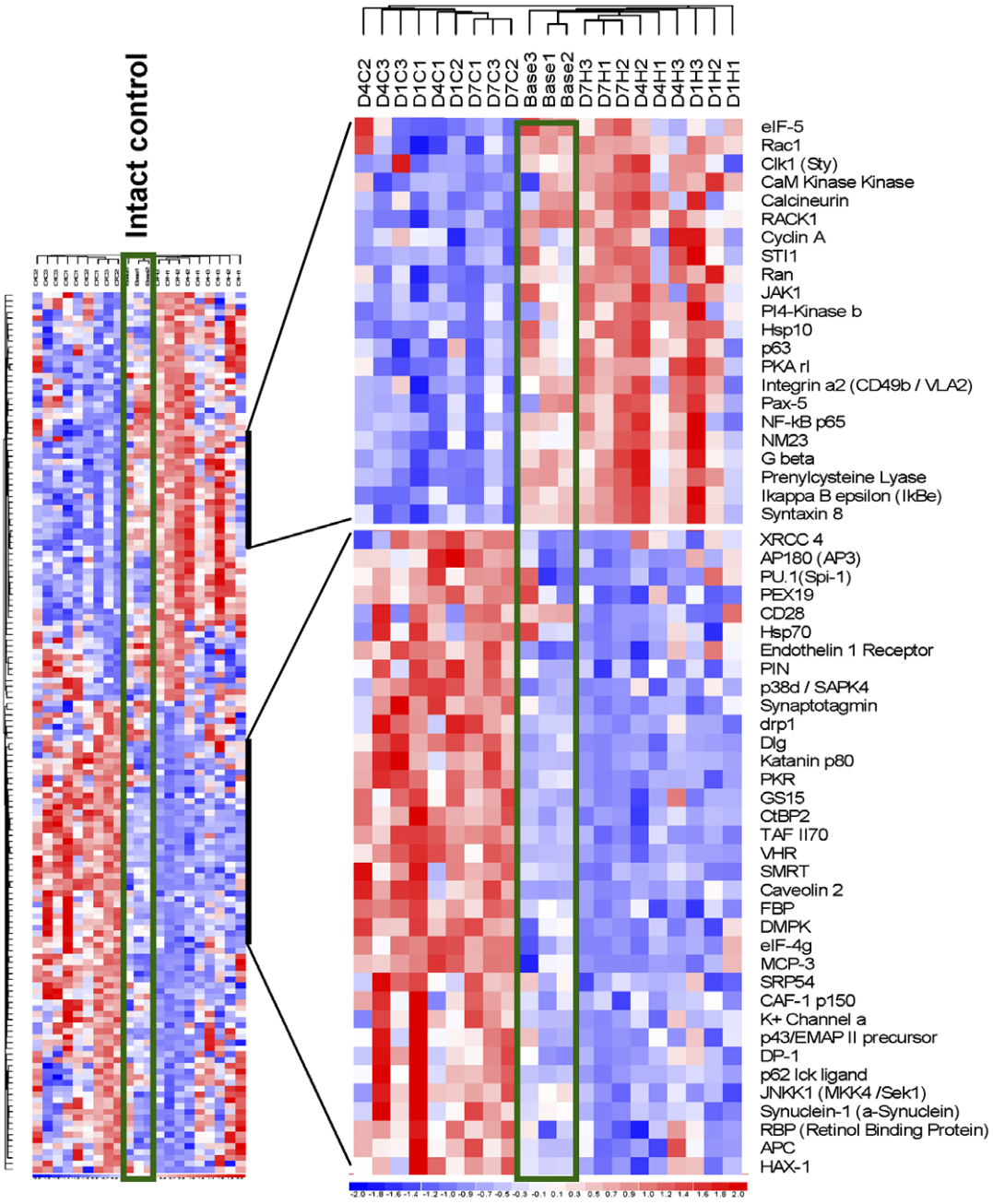

Fig. 3. Cluster analysis using 153 differentially expressed proteins on day 7. All non-treated control samples fell into one cluster while TP508-treated samples and non-fractured control samples fell into another cluster suggesting that TP-508 treatment can reverse the injury profile back to normal physiological profile. The right panel is magnified regions of interest.

of regulatory pathways that are initiated within hours or days post-tissue injury. The early intervention could have a significant impact on overall tissue repair process, whereas intervening with tissue repair enhancement agent in the middle or end stages of a tissue repair process unlikely yield any significant acceleration or improvement [17–19]. As we show herein, TP508 promotes bone healing at the early stage of injury by regulating cell cycle, cellular growth and proliferation, and cell death pathways. Specifically, TP508 stimulates the expression of proteins that promote cell cycle progression, cell survival, and cellular growth to ensure a balance of survival signals over death signals.

A small number of common changes across three early time points was a little surprising, but was reasonable in consideration of their unique cell populations and corresponding physiological process. On day 1, the multiple inflammatory cell types exist mainly in the hematoma including monocytes, macrophages, and neutrophils. These cells provide and respond to cytokines creating the first inflammatory environment critical for fracture healing [20,21], while on day 4 soft callus forms around the fracture

Table 1
Genetic networks with significant score above 20

Network	Analysis	Proteins in network	Score	Focus genes	Top functions
1	D4L vs D4C	BIRC4, BUB3, CASP8, CAV1, CCND1, CDC37, CDKN1A, CRK, CSF2, DIABLO, DOK2, EGFR, FADD, FUS, IRS1, KHDRBS1, PARP1, PIK3R1, PRKCB1, PTPN6, PTPN11, RAD50, RAF1, RAG2, RBL2, RELA, RPS6KA1, SCYE1, SPI1, STMN1, TAF6, TNIK, TRADD, TRAF2, XRCC5	68	35	Cell death, cancer, cellular growth and proliferation
	D7H vs D7C	AP3D1, CCNA2, CD3Z, CDC27, CDK2, CDKN3, CDKN1A, CHUK, E2F2, EIF2AK2, FKBP5, FOS, HSPCA, ITGA2, MAP2K4, MAPK13, MCM4, MLH1, MSH2, NCOR2, NFKBIE, PARP1, PPP5C, RAD50, RELA, SCYE1, STIP1, STK4, STX8, TAF6, TEBP, TFDP1, TP53BP2, TP73L, TRAF2	67	35	Cell cycle, cell death, cancer
	D1H vs D1C	BIRC4, BIRC4BP, CCL2, CCND1, CDC20, CDC25C, CDKN1A, CRI2, DHFR, DLG1, DUSP3, EIF2AK2, EP300, GATA5, HIF1A, ILK, ING4, ING5, MLH1, MSN, MYOD1, NFATC2, NOS2A, PDGFRB, PRKCA, PTGS2, PTPN1, PXN, SMAD4, SNX2, STAT2, TAF6, TEAD1, VGLL4, ZAP70	60	29	Cell death, cancer, cellular function and maintenance
2	D7H vs D7C	AKAP5, APC, ARNT, CAV1, CAV2, CDH5, CNTFR, COPS5, CRK, DLG1, DUSP3, DUSP4, FLOT2, FN1, FUBP1, HDAC3, JAK1, JUP, KIF3B, MAPK3, MAPK8IP1, NFATC2, NOS2A, PIK3CA, PIK3R1, PPP3CA, PTGS2, PTK2B, PTPN1, PTPN6, RPS6KB1, SCARB1, SNCA, SYP, VHL	67	35	Cell death, cellular growth and proliferation, cancer
	D1H vs D1C	ADRB1, ATP2B1, BCL2, BDKRB1, BIK, CABP1, CHUK, CNTN1, CPE, EEF2K, EGF, FYN, GNAT1, HMOX1, IGFALS, IL2, IL1B, INS1, ITPR3, MAP4K3, MAPK13, NCOR2, PAG, PDE5A, SEC8L1, SIPA1, SLC9A1, STMN1, SYT1, TAB3, TAOK3, TNF, TNFRSF4, TRAF2, TREM2	24	15	Cell signaling, molecular transport, small molecule biochemistry
	D4H vs D4C	ADCYAP1, ARHGAP1, ARHGDIA, ARHGEF6, ARHGEF7, ARL6IP, ATRN, BNIP2, CALD1, CDC42, CDC2L1, CHD3, CTCF, CTNNA1, CTSL, FUBP1, GPI, H2AFZ, JUP, MAP3K4, MYC, NME1, PEG3, PEX19, PRKACB, PRKAR1B, PRKAR2A, SIAHBP1, STK6, STMN1, TANK, TP53, TRAF4, UBE2C, UBE2S	21	16	Cellular assembly and organization, cancer, cell cycle

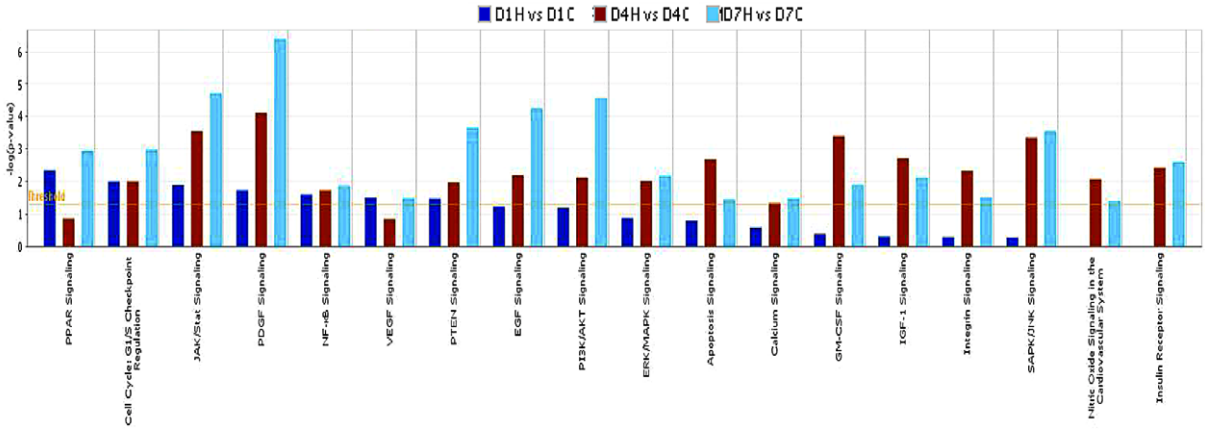

Fig. 4. Significantly enriched pathways in at least two of the three early time points (days 1, 4, and 7). *Y* axis is $\log_2$ transformed significance. $p = 0.05$ is equivalent to y value of 1.3 (horizontal line). The bars from left to right for each pathway represent inflammation (day 1), intramembranous ossification (day 4), and chondrogenesis stage (day 7), respectively.

site and osteoblast progenitor cells in the inner layer of periosteum differentiate and synthesize provisional bone matrix. Cell populations on day 7 are quite different from the previous time points. Chondroblasts, osteoblasts, and fibroblasts become abundant, while osteoclasts have started to resorb the newly formed trabecular bone. Thus, a different group of proteins is required to direct the unique cellular processes on days 1, 4, and 7.

However, those different groups of proteins at different physiological stages have similar functional characteristics. Functional classification, network analysis, and pathway analysis consistently reached one conclusion, that TP508

altered the expression profile of proteins that are functionally related to cell cycle, cellular growth and proliferation, and cell death. Apparently, certain physiological stages require different groups of proteins to function in specific cell populations but to ultimately achieve the same goals; with these proteins being physically interrelated and functionally overlapped (Supplementary Figure 2).

Many of the TP508-altered proteins in the above three functional categories have been shown to be involved in regulating osteoblast/osteoclast activities. Increased expression of PI3KR1, CAMKK1, Fibronectin 1, NFATC1, NFATC2, VHL, and Calcineurin (PPP3CA) accelerate osteoblast formation while enhanced expression of NOS2A and PTGS2 (COX-2), and decreased expression of Dynamin 2 and Caveolin 1 reduce bone resorption. Many other proteins falling into these three categories are implicated in cell survival signals. Well documented proteins that were stimulated by TP508 include RAC1, RELA, CDK2, NF-κB, BIRC4, TRAX, CCNA2, PIK3R1, c-Raf, CSF2, and CCND1. However, some other up-regulated proteins promote cell death, such as BAK1, BIK, CASP8, CASP14, DIABLO, FADD, and TRADD. The up-regulation of those proteins was important in maintaining a balanced cell growth over cell death.

Phosphorylation/dephosphorylation is one of the key regulatory mechanisms in cell signaling. Up-regulation of kinases and down-regulation of phosphatases were prominent phenomena for the differentially expressed proteins falling into the cellular growth & proliferation on day 4. The 11 up-regulated kinases (CDKN1A, PRKAR1B, PRKCB1, RAF1, PRKAR2B, NME1, PIK3R1, PDGFRB, RPS6KA1, PRKAB1, CDC2L1) regulate many key proteins involved in cell cycle and cell growth, such as CDKN1, CDKNA1, cyclin A, RAF1, VEGFA, RAC, BAD, BCL2L1, AKT, MAPK3, NFKB, ERK, PDGFRB, STAT3 and 5, and P58, and also participate in many critical pathways activated in bone healing, including G1/S checkpoint regulation, G2/M DNA damage checkpoint regulation, calcium signaling, and signaling through IGF1, VEGF, TGF, FGF, EGF, PI3K/AKT, PTEN, and integrins. These results suggest that TP508 accelerated bone healing by regulating protein phosphorylation at the critical stage of intramembranous ossification.

That TP508 treatment can reprogram the "broken" expression profile and restore it to the "intact" expression profile (Fig. 3) is a novel finding in this study. The expression profile from intact bone represents a well-orchestrated physiological status, whereas a profile from a fractured bone expresses a stressed environment. Cluster analysis clearly shows TP508 treatment can reverse the stressed profile to normal profile. Creating a physiologically normal environment favorable for bone repair represents another mechanism of TP508 action.

The balance between cell growth and cell death is a fundamental principle in bone healing. Among the 18 significantly enriched pathways, PDGF, VEGF, calcium, and nitric oxide signaling pathways are well known in regulating bone cell growth; NF-κB, IGF-1, and PI3K/AKT signaling pathways are extensively documented in regulating cell survival, whereas apoptosis and PTEN signaling pathways are primarily regulating cell death. However, JAK/STAT and PTEN signaling pathways are not well documented in bone biology and worthwhile examining in greater detail. Both pathways were significant at all three time points in the TP508-treated bone healing, but none of the pathways were significant in any of the three time points during non-stimulated bone healing [15]. Early signals of bone healing are triggered by the immune response, which is regulated by the concerted action of pro- and anti-inflammatory cytokines. JAK/STAT is one major signaling pathway converting the cytokine signal into gene expression programs regulating the proliferation and differentiation of immune cells. Up-regulation of STAT6, PTP1B, PI3K, JAK, and p21cip1 in the JAK/STAT pathway by TP508 can potentially activate transcription of SOCS (suppressors of cytokine signaling), CIS, c-Myc, TIMP-1, Pim-1, α2-macroglobulin, cytokine, and other transcription factors, which ensured a balanced expression of pro-inflammatory and anti-inflammatory plasma cytokine concentrations and provided appropriate immune response triggering bone healing. Functions for PTEN have been identified in the regulation of many normal cell processes, including growth, adhesion, migration, invasion, and apoptosis. PTEN primarily serves to remove phosphate groups from key intracellular phosphoinositide signaling molecules, in particular, PIP3, thereby countering growth and survival signals generated through the PI3K pathway. Thus, PTEN and PI3K signaling pathways cross-talk to coordinate cell survival over cell death. This effort is also apparent in the PTEN pathway where NF-κB was elevated on days 4 and 7, which conveys survival signals, while p21Cip1 was up-regulated at all three time points, which conveys death signals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.07.202.

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