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# Selection of Angiogenic Markers that Predict the Transition from Bisphosphonate Exposure to MRONJ in a Rat Model

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### ABSTRACT

Medication related osteonecrosis of the jaw (MRONJ) is a disorder characterized by loss of blood supply to the jaws and death to the bone. In our previous work, we created a rat model of MRONJ by two injections of 60ug/Kg zoledronic acid (a powerful bisphosphonate abbreviated as ZA) via tail vein followed by extraction of a single first molar. We have shown in this model (ZA-treated rats plus molar extraction) a decrease in the vasculature of the jaws and a delay in bone healing beyond 4 weeks. Purpose: The current study identified angiogenic factors from the jaws of our MRONJ model where expression was altered independent of exposure to ZA alone. Methods: Using RT-PCR arrays containing 84 different gene sequences related to angiogenesis, we screened RNA isolated from the jaws of Control, ZA-treated rats, and our MRONJ rat model (ZA-treated plus first molar extraction), 3 and 6 weeks after extraction. Heat maps of gene expression were analyzed to identify genes where expression was either maximal or lost in MRONJ rats relative to ZA-treated rats without extraction. Results: Our study demonstrates the loss or gain of expression for 22 genes in the MRONJ rat model relative to rats treated with ZA alone. In MRONJ rats, the loss of expression was seen for 10 genes after 3 weeks and 3 additional genes (13 total) after 6 weeks where maximal expression was seen in rats treated with ZA-alone. This study also identified 5 genes that were maximally expressed in MRONJ rats after 3 weeks and an additional 4 genes (9 total) after 6 weeks that were not expressed in rats treated with ZA alone. Conclusions: Our study identifies genes that predict the transition from asymptomatic bisphosphonate exposure to MRONJ.

**Keywords:** Osteonecrosis; MRONJ; BRONJ; animal model; bisphosphonate; markers

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## Introduction

Bisphosphonates are powerful compounds used to treat osteoporosis and the complications of bone metastasis characteristic of certain cancers. Major drug groups such as bisphosphonates have been associated for over ten years with the risk of developing medication related osteonecrosis of the jaw (MRONJ) as a side effect [1-3]. This debilitating pathology is associated with the loss or interruption of blood supply, and results in avascular osteonecrosis (Figures 1 & 2).

The inhibition of angiogenesis, a tightly regulated physiological process of new blood vessel formation may explain the decreased vasculature observed during growth and tissue repair when bisphosphonates are present [4]. Angiogenesis is important in wound healing after an invasive dental procedure such as a tooth extraction. Inhibition of angiogenesis by bisphosphonates supports the observation that tooth extraction is a triggering factor in the development of MRONJ [5].

Studies in several laboratories show that bisphosphonates interfere with angiogenesis at multiple levels. A study by Wood *et al* [6] revealed in both *in vivo* and *in vitro* models that zoledronic acid (ZA) exerts a concentration-dependent inhibition of endothelial cell adhesion and migration necessary for angiogenesis. The work of Fournier *et al* [7] also supports ZA inhibition of angiogenesis by reporting the inhibition of capillary-like vessel formation and elongation *in vitro*.

On the molecular level, key angiogenesis pathways depend on the binding of signaling molecules such as vascular endothelial growth factor (VEGF). A retrospective analysis by Vincenzi *et al* [8] detected significant VEGF reduction at 7 and 21 days after initial IV bisphosphonate administration; claiming that VEGF can potentially be evaluated as a predictive marker of MRONJ. VEGF has a predominant role in induction of angiogenesis, and its cascading effects on other signaling

molecules suggests a number of additional related markers with the potential to predict the onset of MRONJ. A study by Thumbigere-Math *et al* [9] discusses some of these additional markers and limitations encountered with their use.

The onset of MRONJ after exposure to bisphosphonates can take many years to manifest itself as exposed bone in the oral cavity. This key feature of MRONJ is often preceded by an increase in microdamage and a loss of mechanical strength [10]. Criteria for the staging of MRONJ include this exposed bone feature even at Stage 1 of the disease. Recognition of the changes in gene expression for markers that precede this alarming symptom would be beneficial to the clinical outcomes for the patient [11].

The objective of this study, therefore, is to identify angiogenic gene expression patterns that are unique to MRONJ. This study uses the rat angiogenesis array developed by Qiagen™ to examine gene expression in maxillary bone from our MRONJ rat model relative to the maxillary bone from bisphosphonate-treated rats without osteonecrosis.

## Materials and Methods

### Experimental Model

The Institutional Animal Care and Use Committee of Western University of Health Sciences, Pomona CA reviewed and approved the experimental protocol used in this study. This protocol was adapted from a previous study by Marino *et al* [12]. Briefly, twelve Sprague-Dawley adult rats (Harlan, Indianapolis, IN, USA), weighing approximately 200g were purchased and provided with food and water *ad libitum* throughout the study. One week after arrival, eight rats were injected with zoledronic acid (6µg/ 10µL/100g rat weight IV based on a human dose of 4mg/65.8Kg body weight.). The additional four rats were injected with an equal volume of saline. The rats were divided into three groups. The saline –injected group was labeled “Control”, four of the zoledronic acid -

injected rats were labeled “ZA”, and the other four zoledronic acid-injected rats, that were to undergo first molar extraction, were labeled as “MRONJ”. Prior to zoledronic and saline injections, all rats were anesthetized with a rodent cocktail consisting of ketamine (100mg/mL), xylazine (20mg/mL) and acepromazine (10mg/mL).

After the onset of deep anesthesia, the right maxillary first molar was extracted from each of the four rats in the MRONJ group only. Three weeks after the first injection, six of the animals were re-anesthetized, (two animals from each group: Control, ZA, and MRONJ). These six rats were sacrificed and the bone tissue harvested as described below. Rats in these groups were labeled 3 week Control; 3 week ZA; or 3 week MRONJ respectively. All other animals were administered a second injection of either saline (Control group), or zoledronic acid (ZA and MRONJ groups).

At 6 weeks after the first injection (the day of first molar extraction for the MRONJ group), the remaining two rats from each of the three groups (Control, ZA, and MRONJ) were euthanized. A summary of the treatment conditions is found in Figure 3.

On the day of sacrifice, maxillary bone tissue was harvested from the area adjacent to the first molar or first molar extraction site in each group and frozen in liquid nitrogen before storage at -80°C.

### RNA Isolation

Frozen bone samples were placed into 1mL of TriReagent (Qiagen, Valencia, CA), prechilled in liquid nitrogen. Bone tissue was disrupted with a Polytron® homogenizer at maximum speed for 45 seconds on ice. Solubilized bone extract was isolated from bone fragments by centrifugation at room temperature for 15 sec at 8,600 X g. RNA was purified from this bone extract using the RNeasy-Plus® Universal Mini protocol following manufacturer instructions (Qiagen). The amount of RNA present in each sample was determined using a NanoDrop®

spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE).

### RT-PCR

Experimental RNA samples were converted into first-strand cDNA using the RT<sup>2</sup> First Strand Kit® (Qiagen). The cDNA was mixed with RT<sup>2</sup> SYBR Green qPCR Mastermix. This mixture was aliquoted into the wells of the RT<sup>2</sup> Profiler PCR Arrays for “Rat Angiogenic Growth Factors”. PCR was performed using the ABI Step One Plus Real Time PCR System. Relative expression was determined using data from the real-time cycler and the  $\Delta\Delta CT$  method.

### Results

Heat maps constructed from Control, ZA-treated, and MRONJ rats showed distinct expression patterns for the 84 genes in the three treatment groups (see Table1). Note in Table 1 that expression is indicated by the color change from red (maximal expression) to green (minimal expression). Housekeeping genes (Actb, B2m, Hprt1, Ldha, Rplp1) and genomic control (RGDC) genes are not included in the 84 angiogenesis-related genes analyzed.

#### *Comparison of expression between ZA-treated rats without extraction vs. Control rats*

After 3 weeks, decreased expression was observed for 56 angiogenesis-related genes in rats treated with ZA without extraction when compared to Controls. The expression of an additional 5 genes was lost after 6 weeks (Table 2).

After 3 weeks, 13 genes that were not expressed in Controls were now maximally expressed in ZA-treated rats (Table 3). No additional genes were expressed at 6 weeks in the ZA rats.

#### *Identification of genes uniquely expressed in MRONJ*

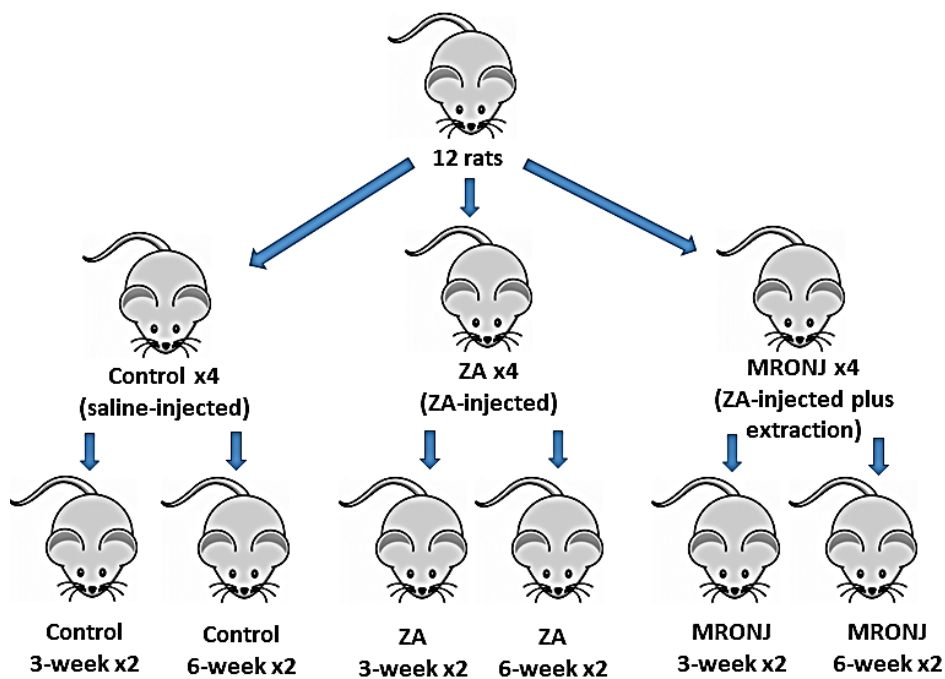
At 3 weeks we identified 60 genes from MRONJ rats, where expression had been lost relative to Control rats. Most of these genes were also lost in ZA-treated rats. Of particular interest, nine of the genes lost in the MRONJ rats were still maximally expressed in ZA rats. These nine genes are listed on Table 4A.



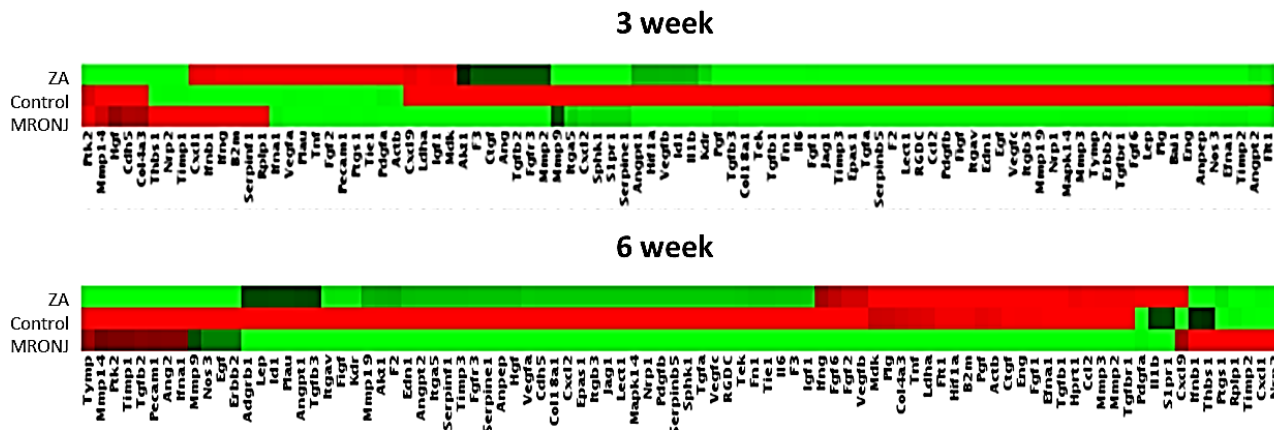
**Figure 1: Exposed necrotic bone in the mandible of a patient with MRONJ.**



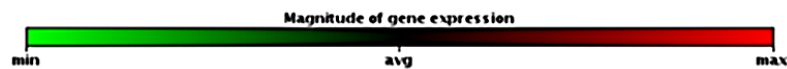
**Figure 2: Panoramic radiograph showing necrotic bone in the mandible and maxilla of a patient with MRONJ.**



**Figure 3: Flowchart of animal treatment groups.**

**Table 1: Heat Maps of Angiogenesis Marker Arrays for Rats\***

\*Note: Genes are not arranged in the same order on the 3 week and 6 week heat maps.

**Table 2 Loss of Expression with ZA vs Controls**

Akt1	Edn1	Flt1	Mapk14	Serpinb5
Ang	Efna1	Fn1	Mmp14	Serpine1
Angpt1	Egf	Hgf	Mmp19	Sphk1
Angpt2	Eng	Igf1*	Mmp3	Tek
Anpep	Epas1	Il6	Mmp9	Tgfa
Bai1 (Adgrb1)	ErbB2	Itga5	Nos3	Tgfb1
Ccl2	F2	Itgav	Nrp1	Tgfb2*
Cdh5	F3*	Itgb3	Pdgfb	Tgfb3
Col18a1	Fgf1	Jag1	Pgf	Tgfbr1
Col4a3	Fgf6	Kdr*	Plg	Timp2
Ctgf	Fgfr3*	Lect1	Ptk2	Timp3
Cxcl2	Figf	Lep	S1pr1	Tymp
				Vegfc

(\* indicates loss of expression first seen at 6 weeks expression of all other genes lost at 3 weeks)

**Table 3: Identification of genes expressed after 3 weeks in ZA-treated rats without extraction but not in Control rats**

Cxcl1	Ilfng	Ptgs1	Vegfa
Fgf2	Pdgfa	Serpinf1	
Ifna1	Pecam1	Tie1	
Ifnb1	Plau	Tnf	

**Table 4A: Identification of genes expressed after 3 weeks exposure to ZA but not expressed in MRONJ rats**

F3	Hif1a	Kdr
Fgf2	Id1	Mmp2
Fgfr3	Igf1	Vegfb

**Table 4B: Identification of genes expressed after 6 weeks exposure to ZA but not expressed in MRONJ rats**

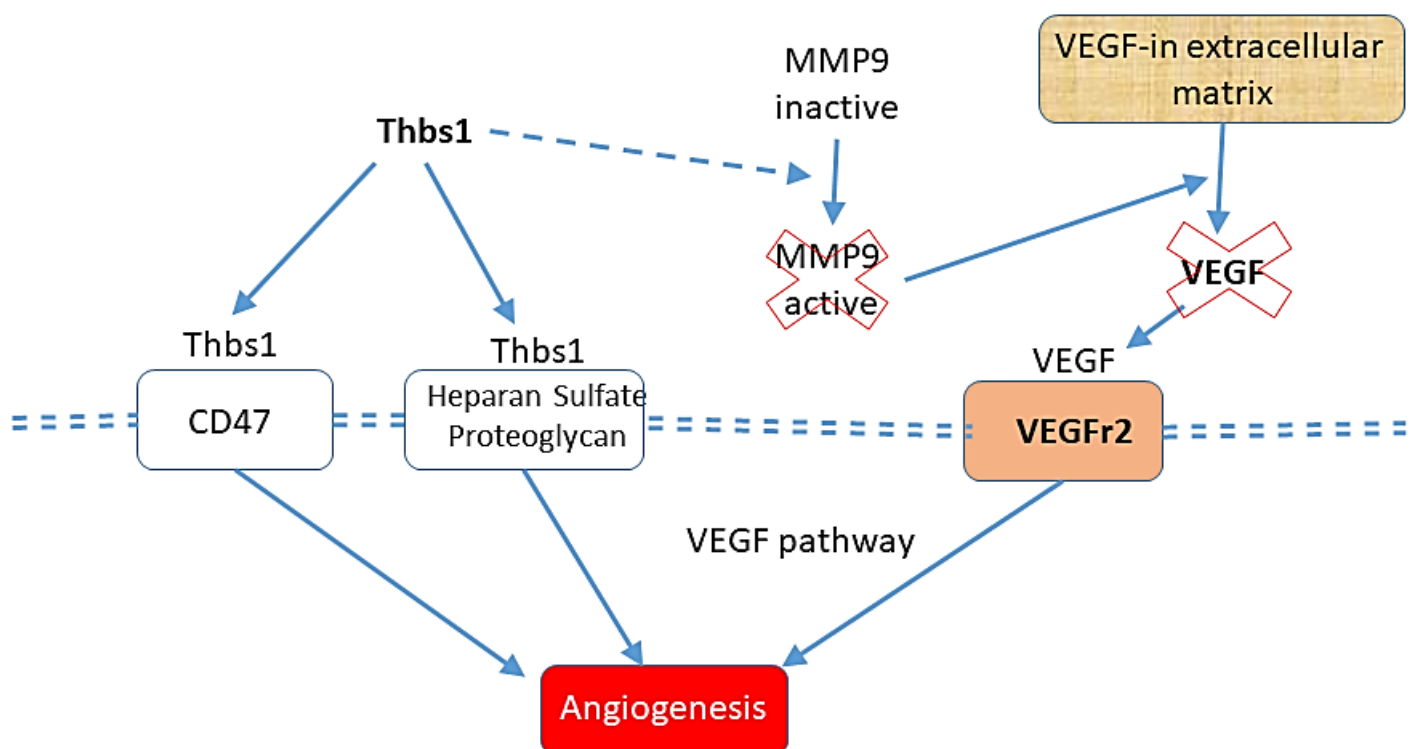
Cdh5	Col4a3	Hgf
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**Table 4C: Identification of genes expressed after 3 weeks in MRONJ rats but not in ZA-treated rats without extraction.**

Nrp2	Thbs1	Timp1
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**Table 4D: Identification of genes expressed after 6 weeks in MRONJ rats but not in ZA-treated rats without extraction.**

Ifna1	Ifnb2	Pecam1	Ptgs1
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**Figure 4: VEGF release from the extracellular matrix by MMP-9 is inhibited by thrombospondin-1**

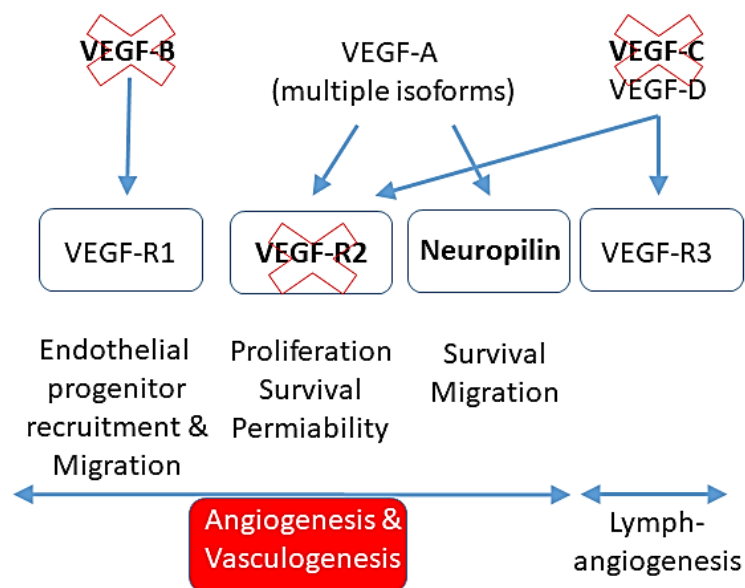


**Table 5: Summary of Results with a List of Possible MRONJ-specific Marker Genes.**

Loss of expression of genes in MRONJ rats but still expressed in ZA-treated rats without extraction		Potential MRONJ-Specific Markers	Genes expressed in MRONJ rats but not expressed in ZA-treated rats without extraction	
6 week	3 week		3 week	6 week
		Cdh5		
		Col4a3		
		Hgf		
		F3		
		Fgf2		
		Fgfr3		
		Hif1a		
		Id1		
		Igf1		
		Kdr		
		Mmp2		
		Vegfb		
		Vegfc		
		Cxcl1		
		Ifng		
		Nrp2		
		Thbs1		
		Timp1		
		Ifna1		
		Ifnb1		
		Pecam1		
		Ptgs1		

Magnitude of gene expression

min avg max

**Figure 5: Expression of Vegfb, Vegfc, and VEGF receptor gene Vegf-r2 (Kdr) are all lost in MRONJ while the expression of the neuropilin gene, Nrp2 is induced**

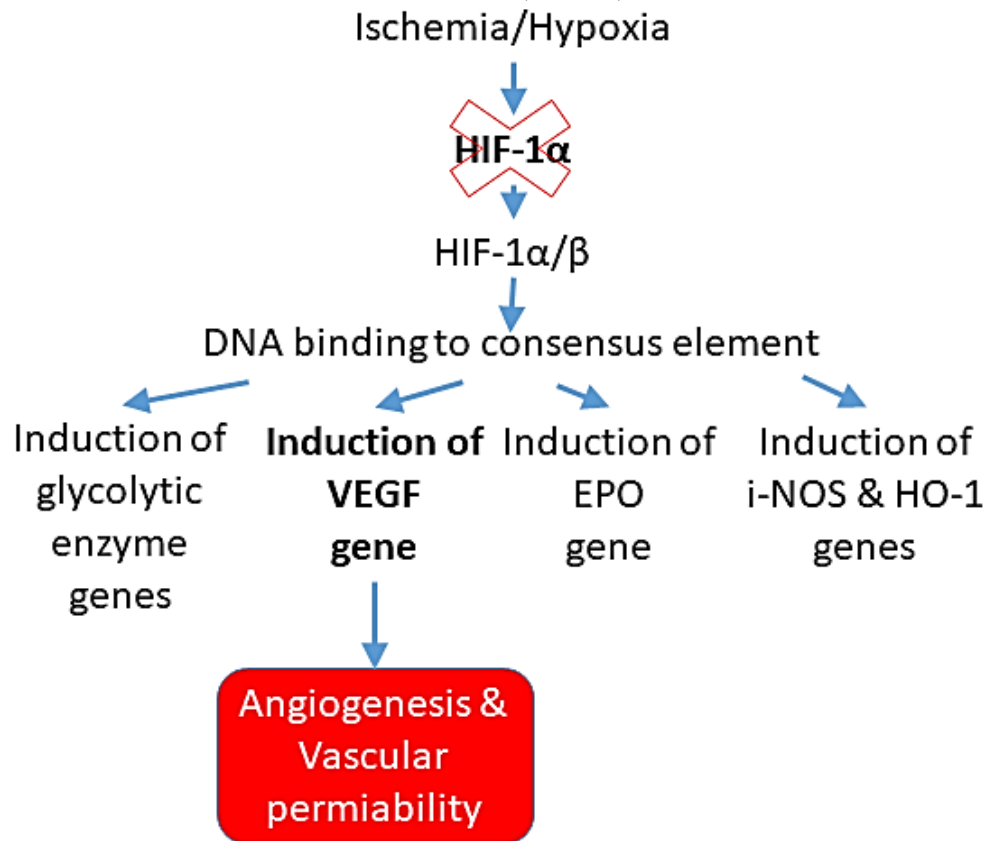


Figure 6: Expression of the Hif1a gene in hypoxic conditions stimulates angiogenesis.

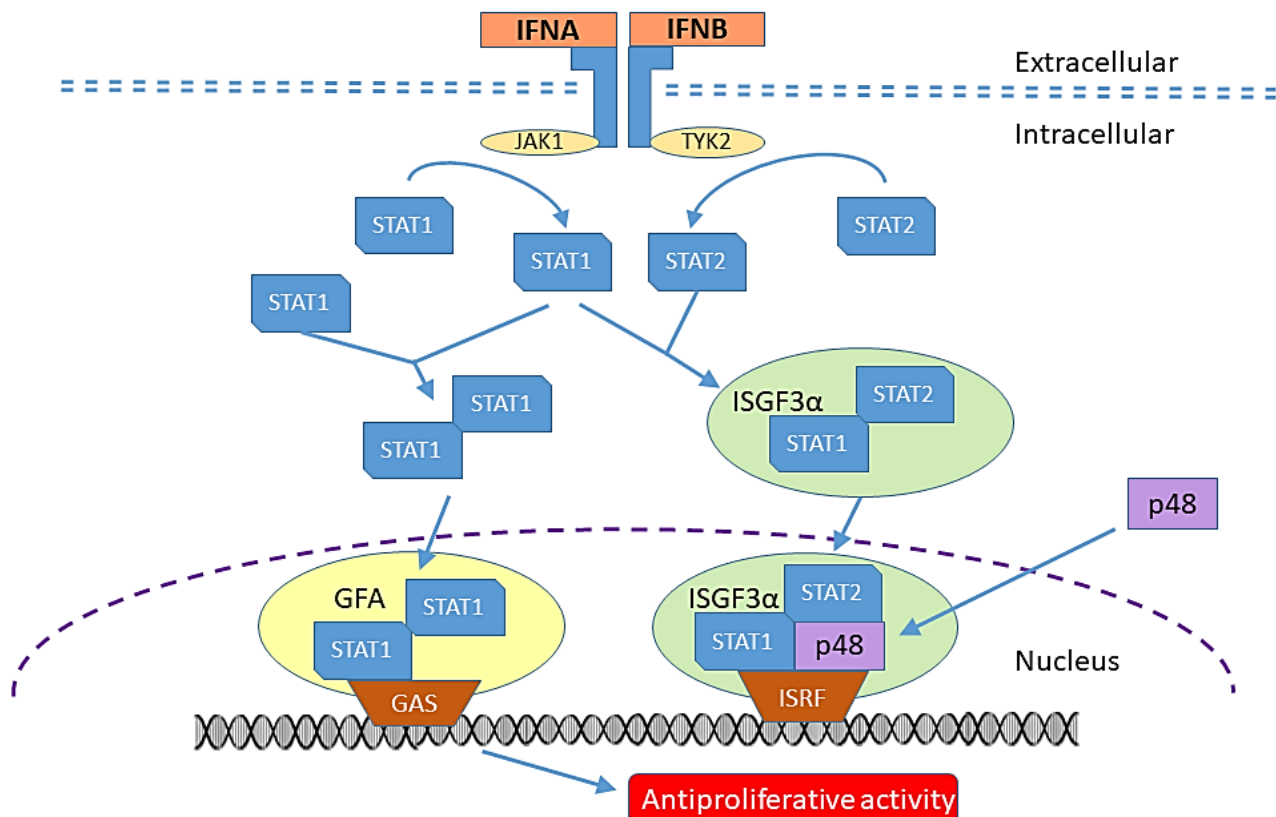


Figure 7: Expression of *Ifna1*, *Ifnb1*, and *Ifng*, are all increased in MRONJ. Binding of interferons triggers the IFN alpha signaling pathway which exhibits antiproliferative properties.



The loss of expression of 3 additional genes was seen after 6 weeks in MRONJ rats that were still expressed in ZA-treated rats without extraction (Table 4B).

After 3 weeks, three genes that were not expressed in ZA-treated rats without extraction were now maximally expressed in MRONJ rats (Table 4C). Four additional genes were expressed after 6 weeks in MRONJ rats that were also not expressed in rats treated with ZA without extraction (Table 4D).

Table 5 represents a summary of these results. The 22 genes listed in the middle column of Table 5 are the potential MRONJ-specific marker genes identified as changing expression (either positive or negative) relative to the expression of the same genes in ZA-treated rats without extraction.

### *Discussion*

After treatment of rats with ZA for three weeks, the early gain of expression of 13 genes (Cxcl1, Fgf2, Ifna1, Ifnb1, Ifng, Pdgfa, Pecam1, Plau, Ptgs1, Serpinf1, Tie1, Tnf, and Vegfa), may be seen as a stimulation of angiogenesis (Table 3). However, the loss of expression of many more genes (61 total) suggests that the overall net effect of ZA exposure even without tooth extraction is to inhibit angiogenesis (Table 2).

While the exact molecular switch has not been identified that explains why gene expression is differentially regulated between zoledronic acid treated groups receiving extraction and groups without surgical intervention, the answer might lie in the examination of how these genes could be related.

Currently, the majority of the differentially expressed genes fit into three different pathways: leukocyte extravasation, FGF2 signaling, and the pro-angiogenic VEGF pathway. One of the normal actions of Cdh5 is to block leukocyte extravasation (13). As levels of Cdh5 drop and levels of Pecam-1 increase, macrophages are able to arrive at the site of injury (14). These macrophages can then activate T-helper cells by secreting factors that

include Cxcl1 (15), Ifn $\alpha$ 1 (16) and Ifn $\beta$ 1, which are increased in the MRONJ extraction group, relative to the ZA group not receiving extraction.

Many of the other differentially expressed genes have upstream and downstream roles in the FGF2 and VEGF pathways. FGF2 has been found to inhibit expression of Thbs1 (17), a potent anti-angiogenic factor, while it binds and activates pro-angiogenic signaling through FGFR3 (17), Hgf (18) and Igf1 (19). FGF2 also appears to increase expression of the tissue factor F3 through EGFR1 (20). The effects of this pathway were likely reversed through the addition of zoledronic acid, which has been shown to reduce FGF2 expression (21), while increasing expression of Thbs1 (22).

The VEGF pathway is another pro-angiogenic system that appears to be differentially regulated in MRONJ animal models. HIF1 $\alpha$  (23) and MMP2 (24) normally help increase VEGF expression and release, however both are reduced in MRONJ models, which could contribute to the reduced levels of VEGF seen. The upregulation of Timp1 may contribute to this reduction, as it has been shown to inhibit MMP2 expression (25). The upregulation of NRP2 may also play a role, as it can act through the SEMA3F receptor to inhibit endothelial cell survival and migration-induced VEGF expression (26). The downstream effects of reduced VEGF can help explain the reduced expression of tissue factor F3 (27) and the type 4 collagen component Col4a3 (28), which are both influenced by the VEGF pathway.

A recent review of the literature suggests that despite a somewhat global down-regulation of gene expression in patients on bisphosphonate therapy, the vast majority of patients (~95 to 98%) do not develop MRONJ [29]. Other studies suggest that impeccable oral hygiene and limited surgical intervention may allow some mitigation of the progression of the disease [30,31]. These observations suggest that it is not only exposure to bisphosphonates that determines the progression of the disease, but also the metabolic activity within the bone and

the surrounding oral and extra-oral tissue environment (including the blood supply) which determine the onset and progression of the disease.

In our study, we analyzed the difference in gene expression of eighty-four angiogenic factors in Control, bisphosphonate-treated (ZA), and MRONJ-induced rats to determine if differences in gene expression could be exploited to identify the transition from bisphosphonate exposure only to MRONJ.

The identification of the twenty-two genes listed in Table 5 suggest this is possible. Of key interest in this Table is the MRONJ-specific expression of thrombospondin-1 (Thbs-1), which is anti-angiogenic through interference with MMP-mediated release of VEGF from the extracellular matrix (Figure 4) [32]. The MRONJ-specific expression of TIMP-2 and the observation that MMP-2 is no longer expressed in MRONJ rats may represent an additional pathway in the anti-angiogenic mechanism.

Of additional interest is the loss of expression at 3 weeks of *Vegfb*, *Vegfc*, and *Kdr* (VEGF-R2 receptor gene). This may explain the upregulation at 3 weeks of *Nrp2* (neuropilin), which codes for a co-receptor for the VEGFR-2 receptor (Figure 5) [33].

Related to this mechanism is our observation that the *Hif1α* gene is highly expressed in rats treated with bisphosphonate but is not expressed after the onset of MRONJ. *Hif1α* is known to induce the expression of more than 60 genes including VEGF in response to hypoxic conditions and other stressors associated with MRONJ (Figure 6) [34].

Another mechanism suggested by our data is the MRONJ-specific expression *Ifna1*, *Ifnb1*, and *Ifng*. Binding of interferons to their receptors triggers the IFN alpha signaling pathway which promotes anti-proliferation of endothelial cells (Figure 7) [35].

Taken together, our data suggests that a specific set of angiogenesis-related markers may be able to distinguish between individuals that are

undergoing bisphosphonate therapy and individuals in the early stages of avascular osteonecrosis prior to the manifestation of frank MRONJ.

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