

DISRUPTION OF APOPTOSIS AND WNT SIGNALING DURING LIMB MORPHOGENESIS BY
EXOGENOUS RETINOIC ACID: A CASE STUDY IN MONODELPHIS DOMESTICA

BY

ANNA CATHERINE MOLINEAUX

THESIS

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Masters Committee:

Assistant Professor Karen Sears, Chair
Professor Ken Paige
Assistant Professor Zachary Cheviron

ABSTRACT

Retinoic Acid (RA) is a vitamin A derivative with severe teratogenic properties. RA is essential for normal limb development; but too much RA results in defects such as digit reduction and truncation of the limb. During normal limb morphogenesis, two signaling centers, the apical ectodermal ridge (AER) and the zone of polarizing activity (ZPA), regulate proper proximal-distal (PD) and anterior-posterior (AP) patterning of the limb, respectively. Despite exhaustive research, there is still no consensus as to how RA interacts with these signaling centers, or the other genes involved in the core-regulatory network for limb organogenesis. By manipulating forelimb growth in *Monodelphis domestica*, a novel model system for limb development, this study sheds new light on the mechanisms by which RA disrupts patterning of the AP and PD axes.

Based on the results of this study, I propose that RA induces digit reduction by upregulating apoptotic events within the ZPA, while simultaneously downregulating SHH expression. Truncation of the limbs PD axis may be the result of the disruptive effects of RA on WNT signaling genes. Characterization of the interactions between RA and genes that regulate these developmental processes are described herein.

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INTRODUCTION

The development of the vertebrate limb has been of great interest within the scientific community for many decades; resulting in extensive investigation that has defined our understanding of the fundamental processes of organogenesis [1-10]. The ease with which the vertebrate limb can be manipulated, in combination with the introduction of powerful molecular tools, allows researchers to obtain great insight into the inner molecular workings of morphogenetic systems.

The core-regulatory network for limb development (outlined in Fig. 1) regulates patterning of the limbs anterior-posterior (AP) and proximal-distal axes (PD) by spatially controlled interaction between sonic hedgehog (SHH) with fibroblast growth factors (FGF), bone morphogenetic proteins (BMP), as well as transcription factors such as GLI3 and BMP-antagonist, GREM1 [1,10,11]. During this process, two signaling centers, the apical ectodermal ridge (AER) and the zone of polarizing activity (ZPA) act as organizers for limb patterning. More specifically, the AER, which runs along the distal tip of the limb bud, is responsible for patterning the limbs PD axis; and the ZPA, located at the posterior of the limb bud, patterns the AP axis [1,2,10-15]. As propagation of the limb begins, FGF10 is secreted from the lateral plate mesenchyme, and works together with BMP4 to form the AER [1,10,16,17]. Once established, the AER secretes FGF8, which maintains FGF10 expression, resulting in limb outgrowth [1,10,16]. BMP4, an integral part of the core-regulatory network, activates its own antagonist, GREM1, which in turn shuts down BMP4 activity [1,10]. As BMP4 expression decreases, so does its suppression of FGF4; which allows for SHH upregulation to occur. This increase in SHH

perpetuates the expression of GREM1 and thereby continuing the cycle of the complex SHH/GREM1/FGF feedback loop [1,10,11].

SHH expression is also mediated by a series of transcription factors. Two of these factors, GLI3 (a suppressor of SHH) and HAND2 (a SHH activator), are suggested to have a mutually antagonist relationship [3,18]. The interaction between these two transcription factors has been shown to play a role in positioning SHH and FGF4 signaling within the limb bud [18,19]. ALX4, lies downstream of GLI3, and is another transcription factor suggested to suppress SHH [1,20,21]; however the specificity of these interactions are still not fully understood.

It has been shown that higher concentrations of SHH program cells at the posterior end of the limb bud to become a pinky finger, and low/no SHH results in the development of digits with more anterior identity, such as an index finger or thumb [1,22,23]. Because digit identity is determined by spatial positioning within the gradient of SHH expression, along with length of exposure time to the morphogen [2,11,13,22-24], it is important to characterize the interactions responsible for when and where SHH is expressed in the limb bud. Identifying these relationships could shed light on how developmental defects such as digit reduction or polydactyly occur.

Another crucial piece to the limb regulatory network is a morphogen called retinoic acid (RA), which interacts with the core-regulatory network for limb growth. RA is a vitamin A derivative found naturally within the body that is essential for proper limb organogenesis [25-28]; however, the precise role of RA in this process has remained elusive.

In the late 1980's and early 1990's reports of RA-induced malformations in human children began to surface [29-32]. In 1991, Rizzo et al. described two cases of newborns exhibiting symptoms such as truncation of the radius, ulna, and humerus, one nearly ablated scapula, as well as digit reduction of the hands occurring in both children. In each case documented in the literature, the mother of the affected newborn was either currently or had been administering RA-containing oral acne treatments, such as Accutane® (isotretinoin) [31,32] or Acitretin [29,30]. Although researchers had already been using exogenous RA to induce limb defects in model species such as mouse (*Mus musculus*) and chick (*Gallus gallus*) for many years [33-36], the consistency in reoccurring birth defects humans left investigators with an even greater sense of urgency to characterize the mechanism by which RA influences patterning of the limb. The results of studies performed in mouse and chick bore an eerily similar phenotypic outcome [21,37,38] when compared to RA-exposed human forelimbs (Fig. 2); suggesting strong conservation across species of the mechanism by which RA impacts limb development.

Despite exhaustive efforts by the scientific community, still to this day, no one has been able to pinpoint the precise interactions between RA and the core-regulatory network. The intensely teratogenic effects of RA are indeed perplexing, but the continued ways in which it causes these malformations through development is not surprising, given the overall complexity of limb morphogenesis itself.

In an attempt to bring resolution to the RA controversy, this study has adopted a less traditional model species, one that represents a natural mutant for limb development, the South American, Grey, Short-tailed Opossum (*Monodelphis domestica*). The opossum is truly

novel in the sense that the physical AER of the developing limb bud is highly reduced [9].

Although the opossum maintains a molecular AER, the influence of the signaling center could potentially be reduced greatly from what is exhibited in other amniotes [9]. This makes the opossum a great model for studying limb development, because the mechanisms underlying limb patterning are, in theory, more simplified; potentially making it easier to tease the system apart to better understand the feedback loops comprising the core-regulatory network.

Regardless of the reduction in the AER activity, RA treatments induce the same malformations in the opossum as exhibited in other model species (Fig. 2,E). This implies that RA is not acting on the system through the AER, but rather through a different molecular pathway. Based on new insight provided by this study, I propose that RA induces digit reduction by upregulating apoptotic events in the ZPA, while simultaneously downregulating the expression of SHH. In accordance with previous knowledge of FGF/WNT controls on PD patterning [39-41], I also suggest that truncation of the forelimb skeletal elements occurs due to the disruptive effect of RA on WNT signaling genes.

MATERIALS AND METHODS

***M. domestica* Embryo Staging**

Monodelphis domestica mating pairs were placed together for 5 days and nights. Video recordings were taken and reviewed daily to confirm the exact date and time of copulation. Breeding and animal husbandry was conducted in accordance with protocols described in Keyte and Smith, 2008 [42] and were approved by the University of Illinois, Institutional Animal Care and Use Committee (IACUC). Staging embryonic development was done in accordance with McCrady, 1938 [43] with modifications by Mate et al., 1994 [44]. Control and RA-treated embryos were collected from stages 28-31 (E11.3-E12.2) for genetic analysis and from stage 33 (E13.75) for phenotypic observation.

Treatment & Dissection

Retinoic acid (RA) slurry was made by reconstituting 50mg of Sigma-Aldrich™ all-trans-retinoic acid powder in 294μL of 100% EtOH. Pregnant opossums were weighed before treatment, and then orally administered a dose of 100mg/kg of RA. Crisco™ vegetable oil was added to the RA slurry to reach a total volume of 200μL. Control opossums were administered 200μL of pure Crisco™ vegetable oil. Treatments were administered 12 hours prior to the embryos reaching the desired stage of development. 12 hours post-treatment, females were euthanized using CO₂ and the uterus and embryos were removed all in accordance with regulations laid out by IACUC. 3 litters of control and RA-treated embryos were collected for stages 28-31, in order to construct an mRNA expression profile from limb bud initiation through the process of limb outgrowth. Embryos that were collected to confirm RA-induced phenotypic

variation were treated during stage 26 (E10.75) and dissected during stage 33 (E13.75). Half of each collected litter was fixed in Qiagen RNALater® for RNA stabilization and later extraction, and the other half in 4% PFA (paraformaldehyde) for tissue sectioning. The embryos placed in 4% PFA were allowed to fix in solution for 4 hours, followed by a 15 minute wash of 100% PBS, 3:1 PBS/MeOH, 1:1 PBS/MeOH, and 1:3 PBS/MeOH, and then stored in 100% MeOH. All embryos were stored at -20°C.

RNA Extraction & cDNA synthesis

Forelimbs were removed from stages 28-31 (E11.3-E12.2) *M. domestica* embryos and homogenized using a Fisher Scientific hand-held Tissuemiser. RNA was extracted from homogenized tissue using an Omega E.Z.N.A® total RNA kit according to the manufacturers protocol. RNA samples were stored at -80°C. cDNA was synthesized from RNA using an Invitrogen SuperScript™ III First-Strand Synthesis System for RT-PCR according to manufacturers protocol and stored at -20°C. Nucleotide concentration of RNA and cDNA samples was determined using a NanoDrop® spectrophotometer.

RNA Sequencing

RNA extractions were performed on wildtype and RA-treated *M. domestica*, stage 29-31 embryo forelimbs as outlined above. 3 individual wildtype and RA-treated samples were run for each stage of development. cDNA libraries were generated and prepared for sequencing according to Illumina® manufacturer instructions. Genes that were significantly, differentially

expressed in response to RA were determined using the Reads per kilo base per million (RPKM) method in Galaxy. All gene expression p-values were taken from Galaxy RPKM analysis.

Gene Network Construction

Significantly differentially expressed mRNAs between wildtype and RA-treated *M. domestica* stages 29-31 were obtained from RNAseq data analysis in Galaxy as described above. Ensembl transcript IDs were converted to HGNC official gene symbols and uploaded into the DAVID Bioinformatics Database. The DAVID functional annotation clustering tool was used to group differentially expressed genes based on their ontology. Clusters of genes involved in embryonic limb morphogenesis were selected for network analysis. These clusters included genes involved in angiogenesis, apoptosis, BMP, FGF, Hedgehog, MAPK, and WNT pathways, embryonic limb and skeletal development, RA response, retinol binding, and ossification.

For each of the above listed clusters of genes, expression levels found in wildtype and RA-treated *M. domestica* forelimbs at each stage (29-31) were uploaded into MMC (Modulated Modularity Clustering), in order to obtain correlation coefficients of expression levels. MMC clusters genes into modules based on similarities in expression pattern from stage 29-31 and provides correlation coefficients data. A positive correlation coefficient between two genes indicates a parallel pattern of expression from stage 29-31, whereas a negative correlation coefficient would indicate an inverse relationship in expression across the timeline of developmental stages.

Cytoscape (version 3.0.1) was used to visualize networks (Fig. 3-5) for both wildtype and RA-treated limbs. Edges for each network were color-coded, blue for positive correlations and

red for negative correlations. Edges of weaker correlations represented by dashed lines (0.9-0.95), and stronger correlations (0.95-1.0) represented by solid lines. Average correlation coefficients for each network were obtained from Cytoscape network analysis output. The clustering coefficient of a selected node is defined as the probability that two randomly selected neighbors are connected to one another [45]. In order to make comparisons of the relative integration of the wildtype and RA-treated networks, the distribution of the average clustering coefficients in each dataset were compared using a non-parametric Mann-Whitney Test. This resulted in two distributions (one for the wildtype and one for the RA-treated network), which could then be compared by a non-parametric t-test to determine if the integration of the RA-treated network was significantly different from that of the control network.

Apoptosis Detection

Monodelphis domestica stage 29 wildtype and RA-treated forelimbs, that had been previously stored in 100% MeOH, were rehydrated into 1X PBS, placed in 30% sucrose:1X PBS at 4°C over-night, then frozen in O.C.T. compound (Tissue-Tek®; Sakura Finetek USA). Limb buds were cryosectioned at 5 microns, placed on glass slides and stored at -80°C. A Millipore ApopTag® Fluorescein Direct In Situ Apoptosis Detection Kit (S7100) was used to detect apoptotic activity on both the wildtype and RA-treated limb bud cross-sections. Apoptosis detection was carried out according to the kit manufacturers recommended protocol. Five control and five RA-treated individual limbs were stained for apoptosis detection. The cross-sections were also stained with 4'6-diamidino-2-phenylindole (DAPI) in order to obtain baseline

cell counts for each sample. Cross-sections were imaged using an immunofluorescence microscope on 10X magnification (Fig. 8), and processed in Adobe Photoshop. Apoptotic cells were each individually hand-counted using ImageJ cell counter. Baseline DAPI cell counts for each cross-section were estimated by applying a 5,000-pixel² grid to the photograph in ImageJ, counting the total number of cells within one square of the grid using the cell counter tool, and multiplying by the number of grid squares the sample occupied.

Real-Time Polymerase Chain Reaction (RT-PCR)

Because *GLI3*, *HAND2* and *SHH* expression are mostly diminished prior to stage 29, variation in expression for these genes could not be characterized in the RNAseq analysis. RT-PCR was performed in order to evaluate how RA exposure impacts the expression patterns of these genes. *GAPDH* was used as the housekeeping gene for these reactions. RNA was extracted from *Monodelphis domestica* forelimbs at stages 28-29 (E11.3-E12.2), and converted to cDNA as described above. Oligonucleotide primers were designed using NCBI Primer Blast and constructed by Sigma-Aldrich™. Amplification reactions were run on a 10µL scale, that contained 5µL of 50ng/µL cDNA, 3.23µL of ddH₂O, 1µL of 10mM F/R primer mix, 0.15µL 50mM MgCl₂, 0.1µL of 10mM dNTPs (BioLabs®), 0.02µL Taq DNA polymerase (Invitrogen™), and 0.5µL of 200mM 10X reaction buffer. Reactions were performed with an initial denaturation at 94°C for 5 minutes, followed by 60 cycles (*GAPDH*), 65 cycles (*GLI3* & *SHH*) of 45 seconds at 94°C, 1 minute at 55°C, and 1 minute at 72°C, followed by a final extension of 2 minutes at 72°C in an Eppendorf thermal cycler. PCR products, along with a low molecular weight DNA ladder (BioLabs®), were separated on 1.5% agarose gels, stained with 0.005% ethidium bromide (EtBr)

at 200mV for 35 minutes. Gels were imaged using a BioRad Gel Doc with exposure time of 0.5 seconds, and band intensities quantified using Quantity One[®] analysis software. Each of the quantifications was standardized against the respective band intensity obtained with the same cDNA using the GAPDH primers.

RESULTS

Treatment & Dissection

Pregnant opossums treated with 100mg/kg of RA during stages of limb initiation and outgrowth resulted in embryos with consistent limb malformations (Fig. 2,E). Digit reduction and truncation of the limb were the most common deformities induced, and closely resemble malformations observed in humans and mice exposed to comparable levels of RA (Fig. 2,A&C).

RNA Sequencing and Gene Clustering

In stage 29,30, and 31 of embryonic development, RNAseq revealed a total of 858, 1254, and 996 mRNA transcripts (respectively) that were differentially expressed in RA-treated *M. domestica* forelimbs when compared to wildtype forelimbs. Of these, 138 genes were selected based on their involvement in limb developmental process. When the expression levels of these genes from both wildtype and RA-treated forelimbs were input into the MMC, they were clustered into 12 modules for the wildtype samples and 11 modules for the RA-treated samples. A matrix of correlation coefficients for the 138 genes was obtained.

Gene Network Construction

Results from the Mann-Whitney statistical analysis revealed that there were two networks with significantly different clustering coefficients (Table 1). These networks impacted by RA-treatment included the apoptosis gene network (Fig. 4) and the network of genes

involved in the WNT signaling pathway (Fig. 5). Expression levels for the genes involved in these networks are listed in Table 3. In both cases, RA treatment resulted in a significant reduction of integration within the network; however, integration of the interaction network for the genes involved in limb development was not significantly impacted. For limb development genes, the average clustering coefficient was calculated to be 0.719 for the control interaction network, and 0.795 for the RA-treated network (Fig. 3). In this case, the RA network is slightly more integrated, however, the variation in average clustering coefficients is not significant between the control and RA-treatment groups. These results suggest that RA does not directly impact the genes that control limb patterning, but rather works on them indirectly through apoptotic and WNT signaling pathways.

When comparisons were made among apoptosis genes (Fig. 4), the control network average clustering coefficient was calculated to be 0.812, and the average clustering coefficient for the RA interaction network was found to be 0.634. This suggests that RA exposure significantly reduced the integration of the genes involved in regulation of apoptotic events in the *Monodelphis domestica* forelimb. Figure 6 visualizes the variation of mRNA expression patterns between the control and RA networks; expression values are also listed in Table 2. ALX4 expression was found to be >2-fold higher in stage 29 (p-value: 0.04682), 30 (p-value: 0.00577). RA-treated forelimbs when compared to controls. Similarly, BMP4 levels nearly double during stages 30 (p-value: 0.00158) and 31 (p-value: 0.00106) in response to RA treatment, and DDIT4 also experienced a drastic increase in RA-exposed forelimbs (>6-fold higher at stage 29 (p-value: 7.31×10^{-12}) and 30 (p-value: 1.02×10^{-11}), then >9-fold higher at stage 31 (p-values: 7.94×10^{-12})). Inversely, PRDX2 expression is downregulated to less than half its

normal level at stage 30 (p-value: 0.00510). Lastly, a retinoic acid receptor gene called RARB, was drastically increased by >12-fold at stage 30 (p-value: 1.10×10^{-8}), and then >20-fold higher at stage 31 (p-value: 3.27×10^{-13}).

Evaluation of expression among WNT signaling genes (Fig. 5) revealed significant variation between the control and RA network average clustering coefficients (control=0.792, RA=0.5). This suggests that increased levels of RA significantly reduces the integration of the WNT signaling network in *Monodelphis domestica* forelimbs. Closer assessment of specific mRNAs are visualized in Figure 7, and listed in Table 2. Of these genes, the most notable results are the immense downregulation of FZD8 and FZD9 levels, and the substantial upregulation of LDB1, SFRP2, and WNT3 in RA-treated forelimbs. FZD8 expression drops to less than half its normal level in all stages, but is only significantly reduced in stage 31 (p-value: 0.00013). In control forelimbs, FZD9 expression is greater than 4-fold higher at all stages when compared to RA-treated forelimbs (p-values: stage 29- 0.00302, stage 30- 0.00317, stage 31- 0.00123). LDB1 levels are first reduced in RA-treated forelimbs at stage 29 (p-value: 0.003), and then increases significantly in stages 30 (p-value: 5.63×10^{-6}) and 31 (p-value: 0.0005). SFRP2 expression increases 2-fold in RA-treated forelimbs at all 3 stages (p-values: stage 29- 4.02×10^{-9} , stage 30- 1.01×10^{-6} , stage 31- 0.00991). Lastly, WNT3 is expressed 2-fold higher at stage 29, 4-fold higher at stage 30, and 2-fold higher at stage 31 in RA-treated forelimbs compared to baseline expression levels (p-values: stg 29- 0.05520, stg 30- 9.39×10^{-5} , stg 31- 0.02079).

Apoptosis Detection

RA-exposed *Monodelphis domestica* forelimbs display a significant upregulation of programmed cell death, specifically within the region of the zone of polarizing activity (Fig. 8). The average number of apoptotic cells in the wildtype limb buds (n=5) was 64.2 (2% of the total cell count), whereas for the RA-treated limb buds (n=5) the average value was 249.2 cells (7.6% of the total cell count). Although there appears to be some upregulation of apoptosis throughout the limb bud, there is an evident concentration of increased apoptotic activity in the ZPA, suggesting that RA is somehow acting to regulate cell death through ZPA specific pathways. DAPI cell counts stayed the same between the wildtype and RA-treated samples, confirming consistency in overall limb bud size between control and RA-treatment groups. Cell counts and p-values listed in Table 3.

Real-Time Polymerase Chain Reaction (RT-PCR)

GLI3 is significantly upregulated in RA-treated forelimbs of *M. domestica* stages 28 (p-value: 0.0457) and 29 (p-value: 0.0131), when compared to wildtype. Due to poor amplification and inconsistency between replicate samples, the relationship between RA and SHH has yet to be characterized. This could be because SHH expression is exceedingly low during stage 28 and almost completely shut off by stage 29 [46]. However, because GLI3, a suppressor of SHH, is upregulated in RA-treated forelimbs, we can predict that SHH expression would be reduced. There was no apparent variation of HAND2 expression in response to the addition of exogenous RA.

DISCUSSION

Treating opossums with exogenous RA induces the same phenotypic anomalies as observed in humans as well as other model species when exposed to increased levels of the morphogen[21,32,37]. Because of this conservation, we can speculate that RA impacts limb organogenesis through similar mechanisms across species. If this assumption holds true, the results of this study have the potential to progress biomedical research.

The RA interaction networks constructed for apoptosis and WNT signaling were significantly less integrated than control networks. This could potentially mean one of two things, it could imply that RA acts on certain regions or modules of the network, while others are not affected, or it could mean that RA has a small influence on all the genes within the network, causing an overall decrease in clustering coefficients. Since some, or maybe even most of the variation in expression is due to indirect, downstream effects of RA, it is hard to say with exact certainty why integration within the networks is reduced. Regardless, it is apparent from results presented in this study that RA is sufficient to disrupt apoptotic events, as well as WNT signaling.

The interaction network for limb development genes is not significantly impacted by the presence of additional RA. From this, we can conclude that RA is not directly targeting patterning genes such as fibroblast growth factors or SHH, but instead it has an indirect effect on patterning through upstream targets.

The interaction network for apoptosis is significantly affected by the introduction of exogenous RA, and contains several key genes of interest. The upregulation of apoptosis

promoter genes like ALX4 and DDIT4, in addition to the simultaneous downregulation of apoptosis suppressor PRDX2, supports the increased concentration of apoptotic events evident in the ZPA of the limb bud. ALX4 is a central piece to this puzzle because its not only a gene involved in promoting programmed cell death, but it also plays an important role in AP patterning, via its interactions with GLI3, HAND2 and SHH. The concurrent upregulation of both GLI3 and ALX4 in RA-treatment groups supports previous claims that ALX4 expression is mediated by its upstream promoter, GLI3 [1,20,21]. The increase in levels of these transcription factors, proposed to play a role in SHH suppression, in combination with the lack of variation in HAND2, leads to the assumption that SHH may be significantly repressed in response to RA; although, this interaction has not yet been confirmed. If this assumption were indeed correct, the significant reduction of the morphogen responsible for patterning digit identity may explain the repeated occurrence of digit reduction in all model species overexposed to RA. Further studies, included quantification of SHH expression, as well as in situ hybridization to reveal the spatial expression patterns of SHH, GLI3, and ALX4 will need to be performed in order to substantiate these claims.

Other genes of significance taken from the apoptosis interaction network include BMP4 and RARB. These genes are not only important apoptotic regulators, but they are also involved in other crucial limb developmental process. BMP4, acts to suppress SHH in the core-regulatory network, so its upregulation in response to RA further corroborates the assumption that SHH may be downregulated with RA-treatment, consequently leading to reduced digit number. RARB is a retinoic acid receptor that also functions as a positive regulator of apoptosis. This

gene is dramatically increased in response to RA, again validating that retinoic acid influences limb patterning via its controls on apoptotic activity.

Within the RA-interaction network for WNT signaling, the genes that appear to be most dramatically obstructed are a series of WNT signal receptors. FZD8, FZD9, and LDB1 are significantly downregulated, while other receptors like SFRP2 and WNT3 are upregulated.

Because FGF/WNT interactions are proposed to control PD patterning of the limb[39-41], any disruption, be it up or down regulation, could have the potential to interfere with proper morphogenetic function. However, the specifics of the relationship between RA and WNT signal transducers have not been characterized in this study. Additional research will be needed in order to better understand the precise impacts of RA on the WNT signaling pathway. Iber and Zeller, 2012 [10] suggests that endogenous RA has a mutually inhibitory relationship with FGFs expressed in the AER. FGF8 expression from the AER perpetuates the expression of FGF10 in the lateral plate and thereby promoting limb outgrowth [1,10,16]. If the amount of RA imposed on the system is significantly increased, it could be predicted that the outgrowth of the limb would be impeded, resulting in the truncation of the limb. During the stages of limb outgrowth, FGF10 levels are reduced to less than half of their normal levels of expression in *M. domestica* RA-treated limbs, suggesting that this may be the key to understanding how RA induces truncation of skeletal elements. Future investigations must be performed to characterize the interactions between RA, FGFs, and the WNT signaling pathway, in order to better understand the mechanism by which RA regulates PD patterning of the limb.

FIGURES AND TABLES

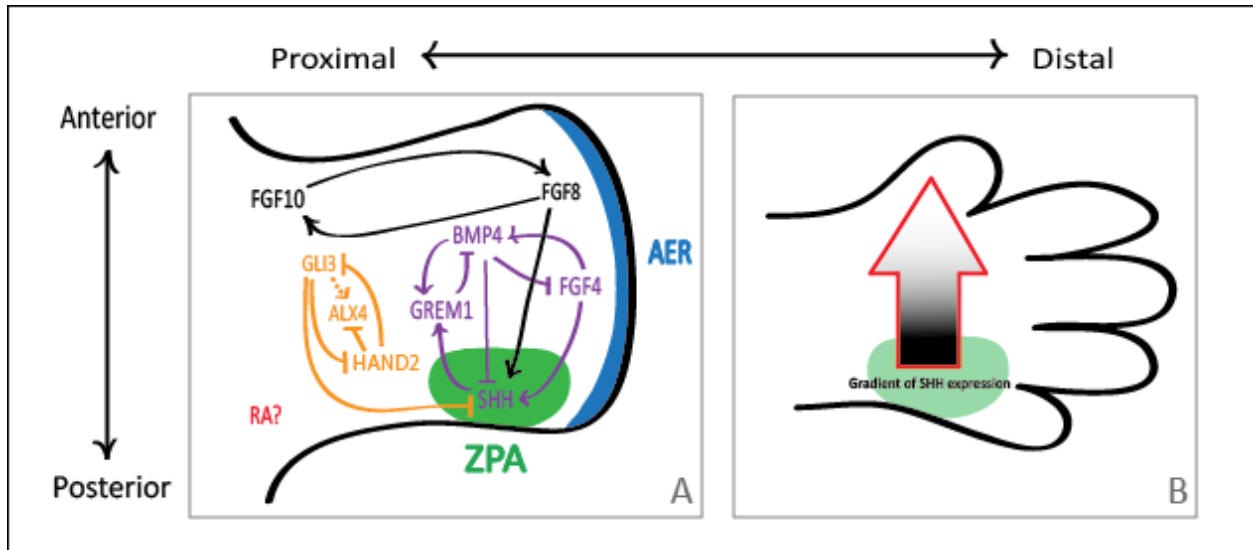


Figure 1: The core-regulatory network for limb development (A). Patterning of the limbs anterior-posterior (AP) axis is established by the zone of polarizing activity (ZPA) located at the posterior of the limb bud [1,2,10-13]; whereas, the proximal-distal (PD) axis is patterned by the apical ectodermal ridge (AER) which runs along distal tip of the limb bud [1,2,10-12]. Digit identity is determined by spatial positioning within the gradient of SHH expression (B) [2,11,13,22-24]. Higher concentrations of SHH at the posterior end of the limb bud result in the formation of a pinky finger, and low/no SHH results in the development of digits with more anterior identity, such as an index finger or thumb.

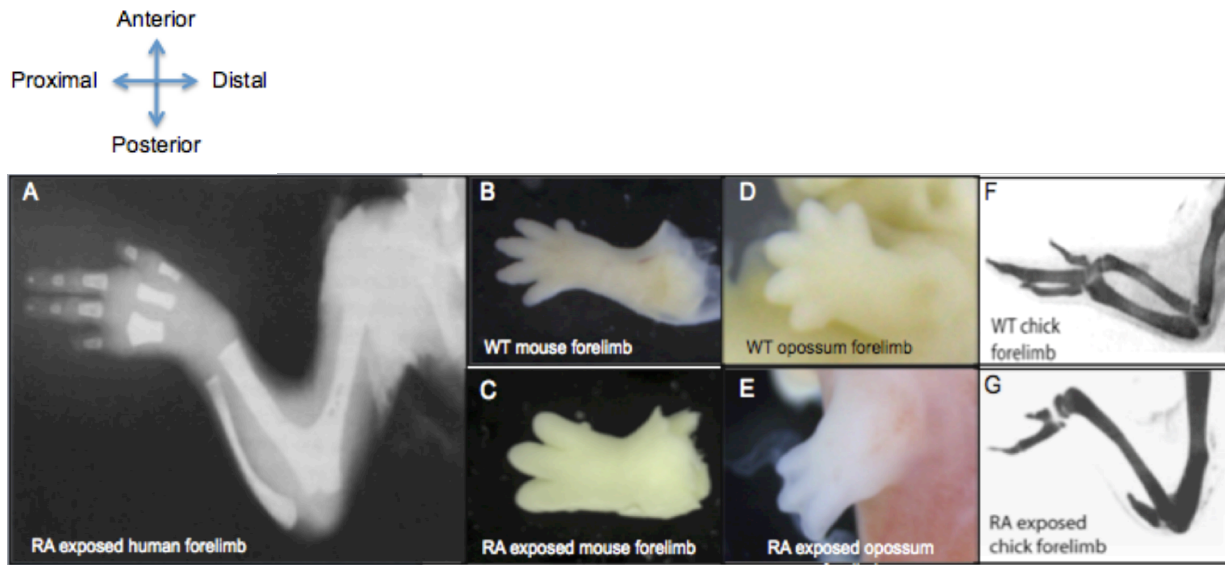


Figure 2: RA-exposed forelimb of a human newborn (A) [29], *Mus musculus* E15.5 (C), *Monodelphis domestica* E13.75 (E) [47] and *Gullus gullus* E10 (G) [37]. Wildtype (WT) *Mus musculus* E 15.5 (B), *Monodelphis domestica* E13.75 (D), and *Gullus gullus* E10 (F) [37]. In all species, RA exposure reduces digit number. In *Monodelphis domestica* and *Mus musculus*, RA exposure also truncates bone length.

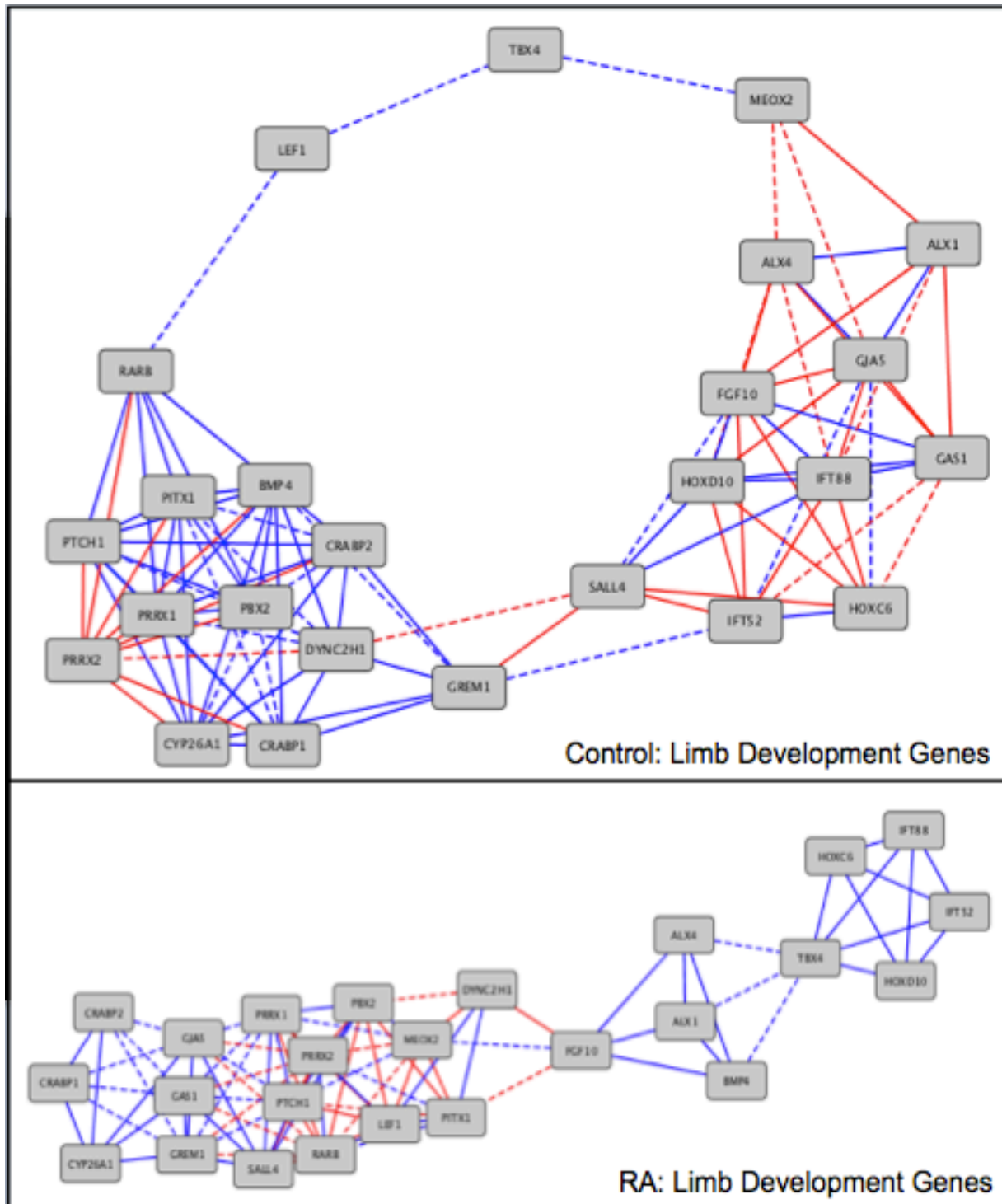


Figure 3: Control and RA-interaction network for genes expressed in the forelimb of *Monodelphis domestica* that are involved in limb organogenesis. Networks constructed based on correlation coefficients of mRNA expression levels from stages 29-31 of embryonic development (E11.7-E12.2). Solid blue lines represent a positive correlation between 0.95-1.0, and dashed blue lines represent positive correlation between 0.9-0.95. Solid red lines represent a negative correlation between 0.95-1.0, and dashed red lines represent a negative correlation between 0.9-0.95. Control network average correlation coefficient= 0.719, RA network average correlation coefficient= 0.795. The RA network is slightly more integrated, however, the variation in average correlation coefficients is not significant, suggesting that RA does not directly impact limb patterning genes, but instead acts through other developmental pathways.

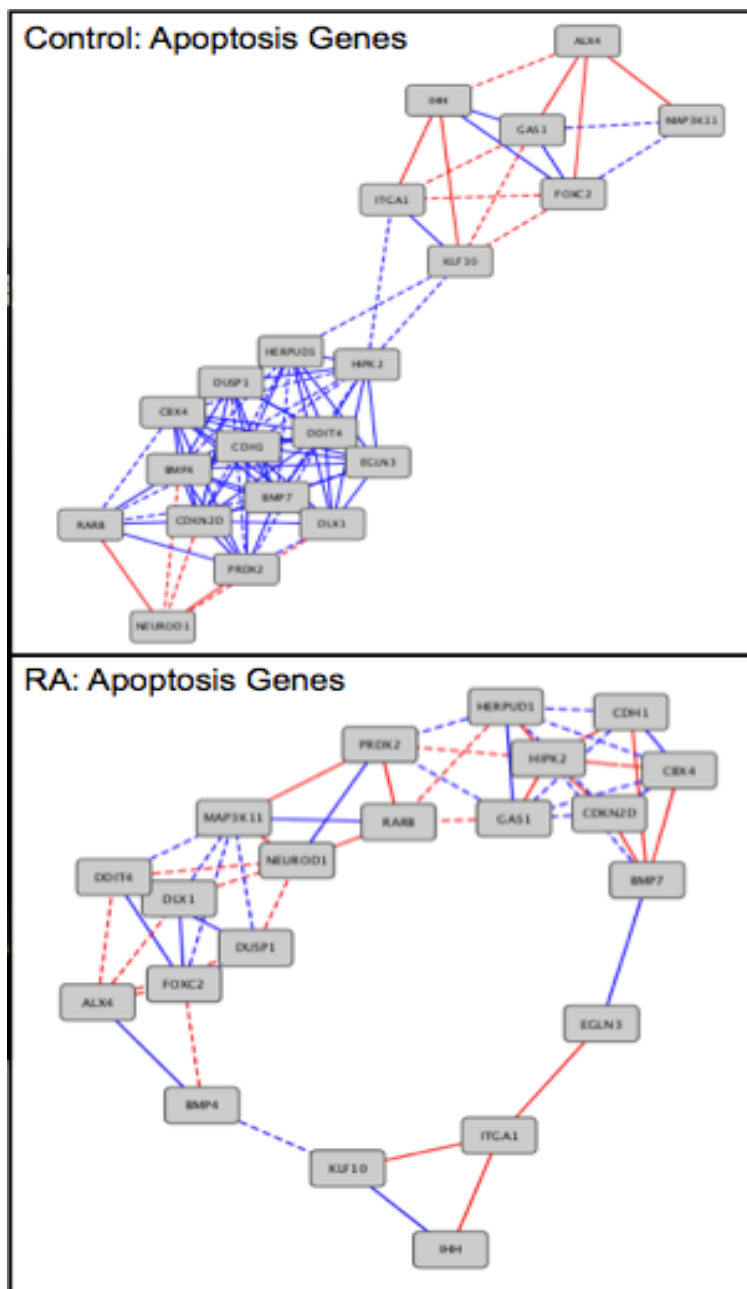


Figure 4: Control and RA-interaction network for genes involved in programmed cell death that are expressed in the forelimb of *Monodelphis domestica*. Constructed based on correlation coefficients of mRNA expression levels from stages 29-31 of embryonic development (E11.7-E12.2). Solid blue lines represent a positive correlation between 0.95-1.0, and dashed blue lines represent positive correlation between 0.9-0.95. Solid red lines represent a negative correlation between 0.95-1.0, and dashed red lines represent a negative correlation between 0.9-0.95. Control network average correlation coefficient= 0.8.12, RA network average correlation coefficient= 0.634. This suggests that RA exposure significantly reducing the integration of the genes involved in regulation of apoptotic events in the *Monodelphis domestica* forelimb.

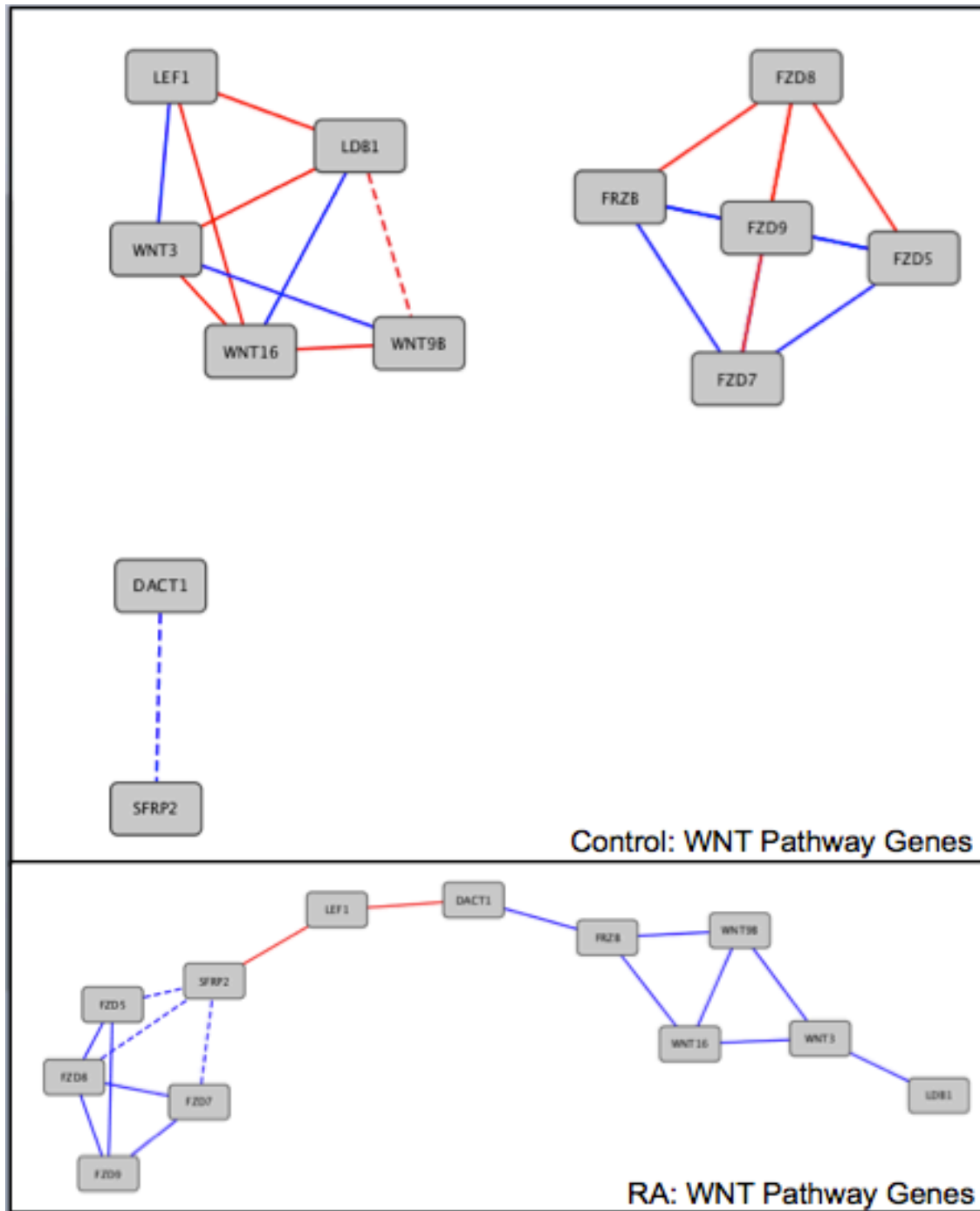


Figure 5: Control and RA-interaction network for genes involved in WNT signaling pathway expressed in the forelimb of *Monodelphis domestica*. Constructed based on correlation coefficients of mRNA expression levels from stages 29-31 of embryonic development (E11.7-E12.2). Solid blue lines represent a positive correlation between 0.95-1.0, and dashed blue lines represent positive correlation between 0.9-0.95. Solid red lines represent a negative correlation between 0.95-1.0, and dashed red lines represent a negative correlation between 0.9-0.95. Control network average correlation coefficient= 0.792, RA network average correlation coefficient= 0.5. This suggests that increased levels of RA significantly reduces the integration of the WNT signaling gene network in *Monodelphis domestica* forelimbs.

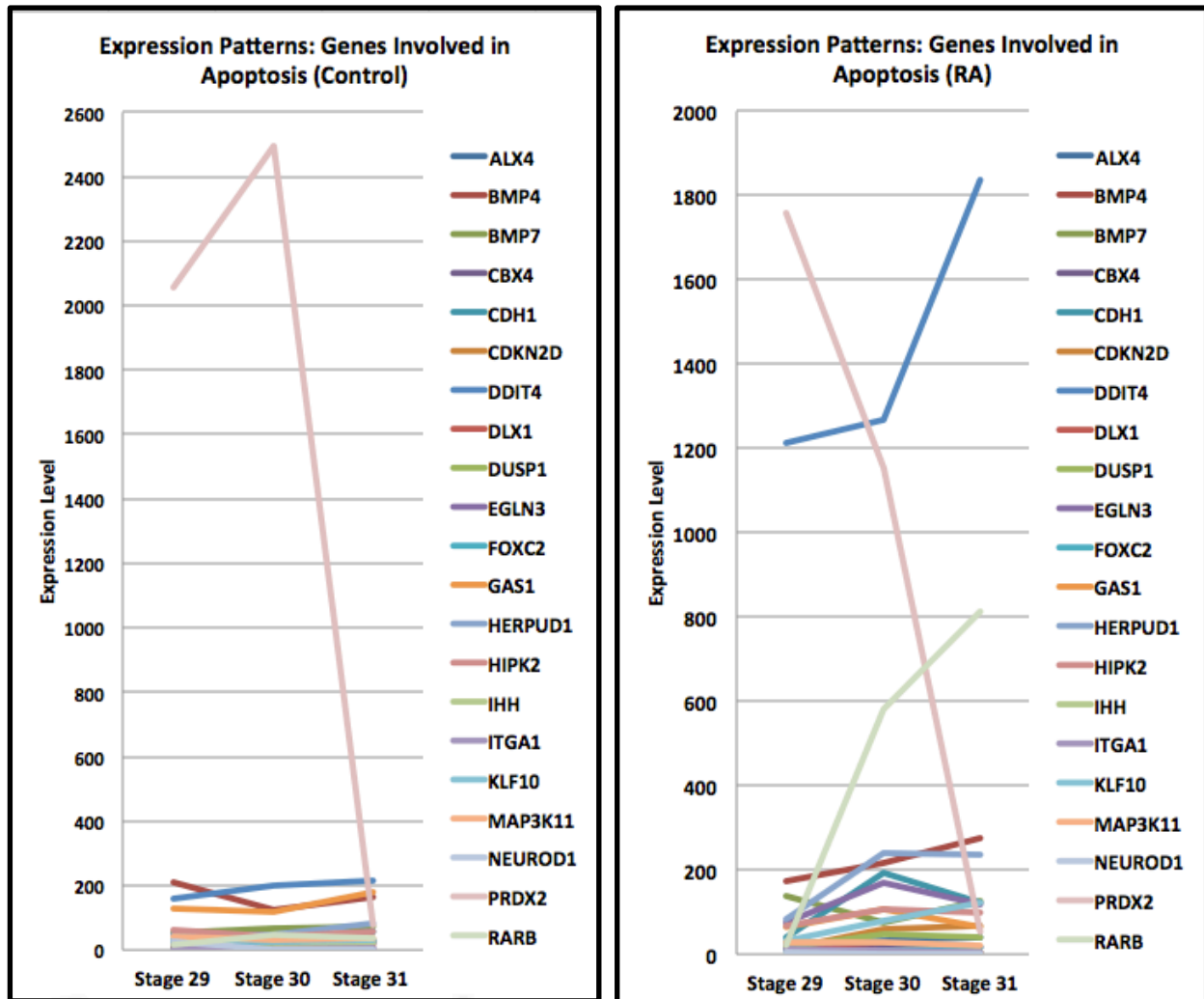


Figure 6: mRNA levels of significantly differentially expressed apoptosis genes, measured in control and RA-treated *Monodelphis domestica* forelimbs. mRNA levels taken at stage 29-31 of embryonic development (E11.7-E12.2). Charts constructed in Microsoft Excel® for Mac 2011 (version 14.3.5) See Table 3 for exact expression levels of each gene.

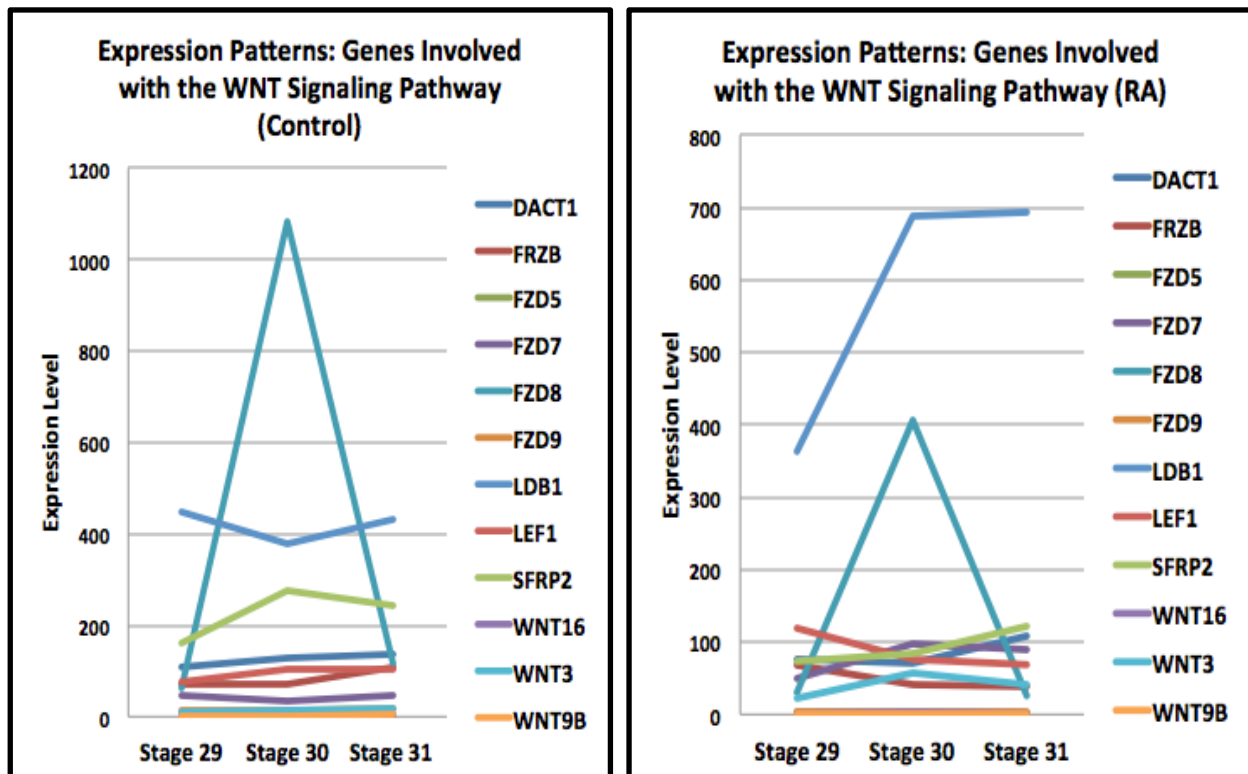


Figure 7: mRNA levels of significantly differentially expressed WNT signaling genes, taken from control and RA-treated *Monodelphis domestica* forelimbs. mRNA levels taken at stage 29-31 of embryonic development (E11.7-E12.2). Charts constructed in Microsoft Excel® for Mac 2011 (version 14.3.5) See Table 3 for exact expression levels of each gene.

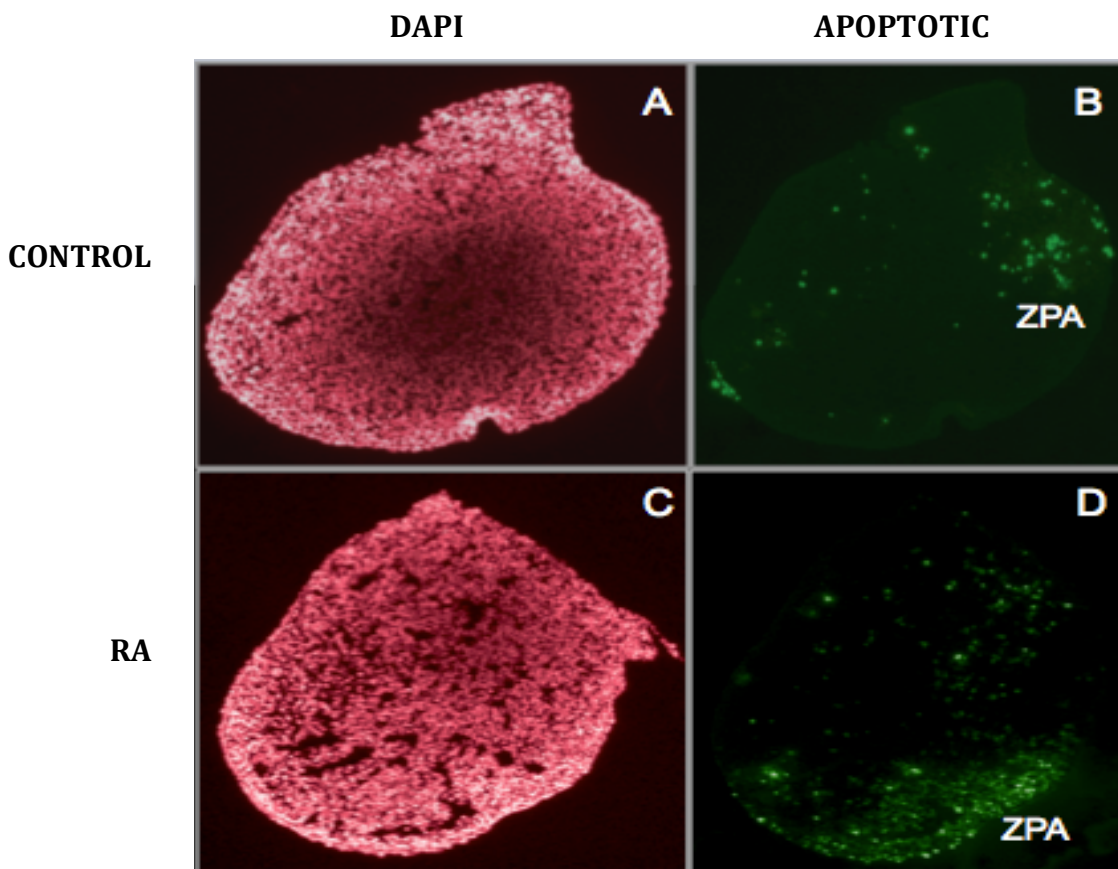


Figure 8: 5-micron cross-sections of stage 29 *Monodelphis domestica* forelimbs imaged using an immunofluorescence microscope on 10X magnification. Panel A&C: Control (A) and RA (C) forelimbs stained with DAPI for baseline total cell count Panel B&D: Control (B) and RA (D) forelimb stained using a Millipore ApopTag® Fluorescein Direct In Situ Apoptosis Detection Kit. Images processed in Adobe Photoshop.

Network	Average Clustering Coefficient	Significant?
Control- All Genes	0.789	No
RA- All Genes	0.763	
Control- Angiogenesis	0.874	No
RA- Angiogenesis	0.757	
Control- Apoptosis	0.812	Yes
RA- Apoptosis	0.634	
Control- Embryonic Skeletal Development	0.731	No
RA- Embryonic Skeletal Development	0.825	
Control- FGF Pathway	1	No
RA- FGF Pathway	1	
Control- Hedgehog Pathway	0.633	No
RA- Hedgehog Pathway	0.563	
Control- Limb Development	0.719	No
RA- Limb Development	0.795	
Control- MAPK Pathway	0.81	No
RA- MAPK Pathway	0.898	
Control- Ossification	0.771	No
RA- Ossification	0.577	
Control- Retinol Binding and Response	0.662	No
RA- Retinol Binding and Response	0.724	
Control- WNT Pathway	0.792	Yes
RA- WNT Pathway	0.5	

Table 1: Results from non-parametric Mann-Whitney statistical analysis of gene networks. Average clustering coefficients calculated by taking the average of all node values for the network.

		RA-Treated			Wildtype		
Gene Network	GENE	Stage 29	Stage 30	Stage 31	Stage 29	Stage 30	Stage 31
Apoptosis	ALX4	24.1646	33.4187	40.9005	11.0194	12.322	23.4759
Apoptosis	BMP4	171.791	214.6092	274.516	211.481	124.24265	166.0394
Apoptosis	BMP7	137.403	73.5812	127.277	52.9885	67.255	72.8488
Apoptosis	CBX4	10.5511	19.9909	17.1532	2.12832	1.388956	3.43919
Apoptosis	CDH1	38.1074	192.61	122.705	18.7204	31.6522	58.6855
Apoptosis	CDKN2D	10.7537	61.0855	68.1636	27.9829	16.1788	27.6506
Apoptosis	DDIT4	1212.03	1269.75	1836.81	158.952	199.221	213.214
Apoptosis	DLX1	21.624	9.86908	4.54708	13.0162	15.8137	18.2071
Apoptosis	DUSP1	14.4416	48.8467	41.343	17.0436	15.8852	30.0162
Apoptosis	EGLN3	73.5274	167.686	116.643	18.0381	25.1515	27.1833
Apoptosis	FOXC2	9.72072	7.55089	16.1603	27.9878	15.4485	26.418
Apoptosis	GAS1	64.6426	108.041	64.5114	129.541	117.581	177.438
Apoptosis	HERPUD1	82.9745	240.86	236.195	47.3972	47.7354	82.1254
Apoptosis	HIPK2	66.9207	105.28	97.8884	60.8835	40.0344	58.1935
Apoptosis	IHH	1.70821	0.877692	3.94424	0.166791	6.91963	22.8685
Apoptosis	ITGA1	10.4053	7.53255	4.0953	3.31356	2.50305	5.52467
Apoptosis	KLF10	31.9128	79.404	121.398	32.1472	25.0086	32.5865
Apoptosis	MAP3K11	27.1037	28.6122	19.5362	40.8414	31.2923	35.2127
Apoptosis	NEUROD1	4.90321	2.06904	0	27.8948	0.539099	0
Apoptosis	PRDX2	1756.99	1154.7	52.8363	2056.63	2497.6	75.6549
Apoptosis	RARB	21.595	580.037	814.345	14.3963	46.6767	38.9581
WNT Pathway	DACT1	76.2532	70.7267	106.885	108.55	128.098	135.942
WNT Pathway	FRZB	68.6198	40.2475	38.5663	72.1849	70.6257	109.288
WNT Pathway	FZD5	3.97937	4.07059	2.18006	0.341864	0.315719	0.514049
WNT Pathway	FZD7	49.5527	98.074	88.3166	45.0129	32.2877	44.3918
WNT Pathway	FZD8	30.8658	405.369	25.8559	62.0047	1083.15	116.387
WNT Pathway	FZD9	2.98163	3.13574	4.39878	13.954	12.4033	17.862
WNT Pathway	LDB1	363.053	687.69	694.129	447.154	377.002	431.548
WNT Pathway	LEF1	117.99	76.8722	68.718	76.0796	104.166	103.359
WNT Pathway	SFRP2	72.3089	84.7909	120.955	159.294	276.521	242.733
WNT Pathway	WNT16	2.17525	3.74176	1.34933	6.20876	5.29011	6.83581
WNT Pathway	WNT3	21.2093	57.5576	41.1459	10.265	13.1344	19.0274
WNT Pathway	WNT9B	0.713037	0.256415	0.433547	1.35068	3.15738	3.99859

Table 2: Expression levels of apoptosis and WNT pathway genes determined to be differentially expressed between wildtype and RA-treated *Monodelphis domestica* forelimbs stages 29-31. Expression data was taken from RNAseq analysis, and input into MMC.

Sample	# Apoptotic Cells	# DAPI Cells	% Apoptotic
Wildtype	78	3577	2.180598267
Wildtype	82	3283	2.497715504
Wildtype	19	2560	0.7421875
Wildtype	87	2860	3.041958042
Wildtype	55	3087	1.781665047
RA-treated	261	3422	7.627118644
RA-treated	114	3136	3.635204082
RA-treated	319	3072	10.38411458
RA-treated	346	3763	9.19479139
RA-treated	206	2774	7.426099495
	Average Apoptotic Cell Count	Average DAPI Cell Count	Average % Apoptotic
Wildtype	64.2	3153.4	2.048824876
RA-treated	249.2	3233.4	7.653465639
P-Value	0.00906778	0.526806246	0.005840538

Table 3: Apoptotic and DAPI cell counts. Each apoptotic cell was hand-counted using ImageJ cell counter. RA-exposed forelimbs display a significant (p-value 0.0091 for average apoptotic cell count, p-value 0.0058 for average % apoptotic cells) upregulation of apoptotic activity. DAPI cell counts were estimated using ImageJ cell counter on a 5,000-pixel² grid and there is no significant difference between wildtype and RA-treated forelimbs.

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