

Review

Molecular Control of Interdigital Cell Death and Cell Differentiation by Retinoic Acid during Digit Development

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Abstract: The precise coordination of cell death and cell differentiation during the formation of developing digits is essential for generating properly shaped limbs. Retinoic acid (RA) has a fundamental role in digit development; it promotes or inhibits the molecular expression of several critical genes. This control of gene expression establishes molecular cascades that enable both the commencement of cell death and the inhibition of cell differentiation. In this review, we focus on the antagonistic functions between RA and fibroblast growth factor (FGF) signaling in the control of cell death and between RA and transforming growth factor beta (TGF β) signaling in the control of cell differentiation.

Keywords: interdigital cell death; retinoic acid; limb development; digit formation; apoptosis; FGF; TGF β

1. Introduction

During formation of definitively shaped embryonic limbs, molecular control of cell differentiation and death directs the number of digits and digit separation. During evolution, the precise control of these processes resulted in the manifestation of a great diversity of appendages in all tetrapod species, which are adapted to the habitat in which they live. The number of digits varies from five to one, and they can be webbed or not. Notably, presumptive digits originate from cartilage condensations that give rise to digital rays that are always separated by interdigital tissue. In species with free digits, cell death occurs in the interdigital tissue. In contrast, in species with webbed digits, interdigital membranes are permanent, because the molecular cascades responsible for cell death are inhibited.

At advanced stages of limb development, cells from the undifferentiated zone underneath the apical ectodermal ridge (AER) either differentiate or die, according to signals that they receive once they move away from the zone [1]. The AER is a specialized region of the ectoderm located between the dorsal and ventral ectoderm that rims the distal margin of the limb bud [2]. The regulation of the maintenance of mesenchymal cells in an undifferentiated state depends on fibroblast growth factors (FGFs) and Wingless-Type MMTV Integration Site Family (WNT) proteins released from AER [3,4]. In embryonic chick limbs, Transforming Growth Factor beta (TGF β)/activin signaling promotes cartilage differentiation and interdigital cell death has been shown to depend on retinoic acid (RA) signaling [5–7]. The integration, in time and space, of these signaling mechanisms is important for directing the definitive shapes of embryonic limbs. The main aim of this review is to summarize the current knowledge on the molecular control of programmed cell death by RA during digit development.

2. Digit Development

The tridimensional organization of limbs during embryonic development depends on three signaling centers of control. First, the AER directs proximal-distal growth. Second, there is the dorsal and ventral ectoderm. Finally, there is the Zone of Polarizing Activity (ZPA), which corresponds to a group of mesodermal cells located at the posterior region of the limb. The ZPA directs anterior-posterior axis formation [8–10]. RA induces the expressions of *homeobox B8 (Hoxb8)* and *dHand*, and then *Sonic hedgehog (Shh)*; this establishes the ZPA [11–14]. ZPA transplants and SHH-soaked beads placed in the anterior region of a limb are able to generate limb duplications with mirror-image symmetry [15]. Once SHH binds to transmembrane receptors of the Patched (Ptc) family, GLI proteins become transcriptional activators instead of transcriptional repressors (as occurs in the absence of SHH signaling). At early stages of limb development, the high activity of SHH in the ZPA is reflected by the presence of the GLI3 activator, whereas low SHH activity is reflected by the presence of the GLI3 repressor [16]. It is well known that *Gli3* mutant and double (*Gli3* and *Shh*) mutant mice experience polydactyly with unpatterned digits [17,18]. Remarkably, in all cases, extra-digit formation is always accompanied with the formation of extra interdigital tissue. These results indicate that SHH/GLI3 signaling controls the digit number.

2.1. Cartilage Differentiation

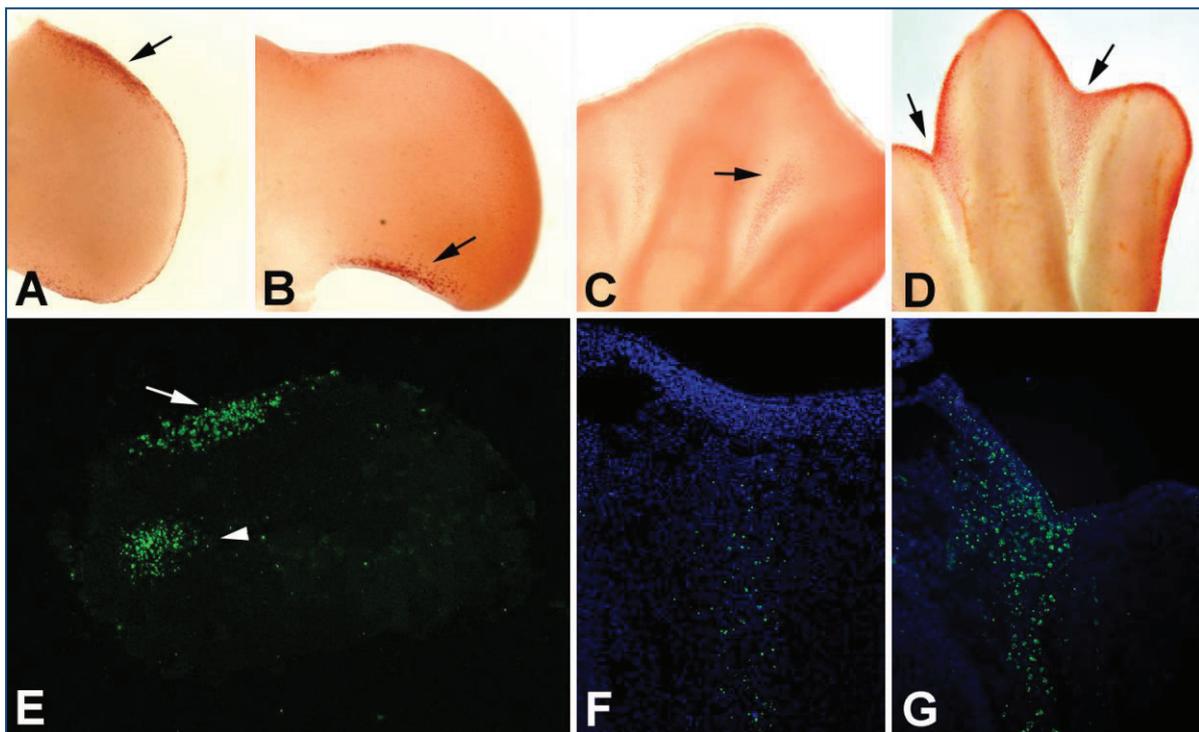
During digit development, cartilage differentiation initiates when mesenchymal cells from the undifferentiated zone are recruited by chondrogenic factors to the chondrocyte lineage. The onset of cell recruitment occurs between the condensed cartilage and the AER, in the boundary of the growing digital region; the recruitment occurs in a zone that has been called either the phalanx-forming region (PFR) or the digit crescent (DC) [7,19]. The PFR/DC consists of mesenchymal cells that are characterized by the expression of *Sox9*, *Bmpr1b*, and *Activin β a* genes. These cells are also positive for pSMAD 1/5/8, which indicates that Bone Morphogenetic Protein (BMP) signaling occurs [7,19]. Members of the TGF β superfamily, such as TGF β and Activins, are also expressed in these digit regions. They are able to trigger the molecular cascade that stimulates the formation of cartilage tissue, because they induce the expression of *Sox9* [20–23]. BMPs are able to maintain *Sox9* expression at the tip of the developing digits in the PFR/DC, but are unable to recruit the cells underneath the AER for chondrocyte development. Instead, BMPs promote massive cell death.

Interdigital tissue is destined to either die or become permanent. This depends on whether the molecular cascade for cell death is activated or not [6,24,25]. Moreover, cell death has an important role in establishing digit identity [26]. In chick embryos, digital identity is associated with the number of phalanges. Members of the BMP family in interdigital tissue control the different levels of SMAD1/5/8 activity in the PFR/DC; these activities determine digit identity [7,26]. Remarkably, if interdigital tissue is removed and cultured *in vitro* (or disaggregated) and the resulting cells are cultured as a micromass, the fate of the tissue is changed. The tissue either forms cartilage that resembles a digit or the cultured cells differentiate generating cartilage nodules (instead of dying) [27]. These results suggest that interdigital tissue is able to become cartilage. Also, when a TGF β -soaked bead is implanted, the tissue is able to grow an ectopic digit as a consequence of the molecular cascade (chondrogenesis) initiated by TGF β . Under these conditions, the inhibition of cell death and the induction of *Sox9* expression occurs [21,23,27]. Interestingly, formation of cartilage is antagonistic to the initiation of cell death. Although the formation of digits and digit separation requires the crosstalk of several signaling molecules, in this review, we will focus on the roles of the antagonism between RA and FGF and the antagonism between RA and TGF β signaling in the control of cell death and cell differentiation.

3. Programmed Cell Death in the Limb Mesoderm

Apoptosis is the most abundant type of cell death that takes place during limb morphogenesis. Spatio-temporal control of apoptosis during morphogenesis in mesenchymal cells is an important mechanism for shaping limbs [28]. In the embryonic limbs of avians, apoptosis is observed in the anterior and posterior margins of the limbs, and in the interdigital tissue. Although apoptosis has been observed in these regions, they have traditionally been called the anterior, posterior, and interdigital necrotic zones (ANZ, PNZ, and INZ), respectively, because they were recognized before the identification of apoptosis. However, with the current status quo, these areas should correctly be called the anterior, posterior, and interdigital apoptotic zones (AAZ, PAZ, and IAZ), respectively (Figure 1) [28–30].

Figure 1. Regions of cell death during chicken limb development. (A) Hind limb bud at stage 22HH showing the anterior apoptotic zone (AAZ) (arrow). (B) Anterior limb bud at stage 24HH exhibiting the posterior apoptotic zone (PAZ) (arrow). (C,D) Cell death is observed in the interdigital apoptotic zone (IAZ) of the hind limb at stages 31HH and 32HH, respectively (arrows). Neutral red staining is shown in A–D. (E) Tissue section of hind limb at stage 23HH demonstrating the presence of the Patch Opaque (arrowhead) and the AAZ (arrow), which are TUNEL-positive. (F,G) Tissue sections from the interdigital regions of hind limbs at stages 31HH (F) and 32 HH (G) exhibiting apoptotic cells (as revealed by the TUNEL assay). In (E), the proximal region is at the left, and in (F) and (G), the proximal regions are at the bottom.



Molecular control of apoptosis occurs through at least two broad pathways, an “intrinsic” and an “extrinsic” one [31]. In both pathways, signaling results in the activation of caspases. In the intrinsic pathway, once cell death signals are triggered, proapoptotic BH3-only proteins (BID, BIM, and PUMA) activate BAX and BAK proteins to promote the release of cytochrome c from the mitochondria into the cytosol, thereby inducing apoptosome formation [32]. The apoptosome involves the interaction of cytochrome c with dATP, APAF-1, and procaspase 9; these interactions activate caspase 9. Caspase 9 in turn activates executioner caspases, such as caspase 3 [31]. Also, the activation of caspases 9 and 3 occurs when the protein SMAC/DIABLO, released from the mitochondria, inhibits Inhibitors of Apoptosis (IAP); IAP inhibits caspases [33,34]. Likewise, apoptosis inducing factor (AIF) and endonuclease G (endoG), two proteins released by mitochondria, are translocated to the nucleus to promote chromatin condensation and the formation of high-molecular DNA fragments [31]. In this situation, no cytochrome c-mediated activation of caspases occurs [32]. The inhibition of apoptosis occurs by members of the BCL2 family proteins. Under survival conditions, these proteins bind to BAX or BAK to avoid the release of cytochrome c from mitochondria.

The extrinsic pathway of apoptosis is triggered by the activation of death receptors by their specific ligands. This leads to caspase 8 activation. As an initiator caspase, caspase 8 activates caspase 7 and caspase 3 via proteolytic processing. In addition, caspase 8 promotes the activation of BID, which together with BIM and PUMA, induces the release of cytochrome c from mitochondria [32]. Cathepsins are also involved in the extrinsic pathway of apoptosis. They are released into the cytosol after lysosome membrane permeabilization activates BID and degrades BCL-2. The release of cytochrome c from mitochondria is subsequently triggered, which leads to the initiation of the intrinsic pathway of apoptosis [35].

Mice deficient in either *Bax* and *Bak* or in *Bim*, *Bid*, and *Puma* (which code for pro-apoptotic proteins) display syndactyly, because no interdigital cell death occurs [32,36,37]. However, if caspase activity in the interdigital tissue of chick limbs is blocked by broad-spectrum caspase inhibitors, such as Z-VAD-FMK, or in *Apaf1*^{-/-} single mutant mice or in compound mice for caspases, interdigital cell death is not inhibited, suggesting a mechanism of caspase-independent apoptosis [38–42]. On the other hand, it has been demonstrated that the expression of lysosomal cathepsins commits mesenchymal cells to die [43]. Interestingly, blocking the function of cathepsin does not inhibit interdigital cell death (Table 1) [44]. However, it has been suggested that cooperation between the extrinsic and intrinsic pathways of apoptosis during interdigital cell death occurs, because the simultaneous experimental inhibition of both cathepsins and caspases inhibits interdigital cell death [44]. Recently, it was suggested that the canonical caspase pathway leads to an initial activation of neutral endonucleases to initiate interdigital cell death. The second step, the cleavage of Serpin B1, promotes the expression of acidic nucleases, coinciding with interdigital pH acidification during tissue regression. In conclusion, interdigital cell death occurs by the coordinated and sequential activation of the caspase and lysosomal degenerative molecular cascades [45].

Table 1. Phenotypes observed in mutant mice for genes related to cell death or in embryonic chick limbs treated with inhibitors of caspases and cathepsins or both (see text for details).

	Mutant /Treatment	Model	Phenotype	Reference
Apoptosis	<i>Bax</i> ^{-/-} <i>Bak</i> ^{-/-}	Mouse	Syndactyly	[32]
	<i>Bim</i> ^{-/-} <i>Bid</i> ^{-/-} <i>Puma</i> ^{-/-}	Mouse	Syndactyly	[32]
	<i>Apaf</i> ^{-/-}	Mouse	Normal apoptosis	[35]
	Z-VAD-FMK treatment	Chicken	Normal apoptosis	[40]
	Inhibition of cathepsin	Chicken	Normal apoptosis	[44]
	Inhibition of cathepsin and caspases	Chicken	Syndactyly	[44]

Interestingly, interdigital tissue regression also involves intense remodeling of extracellular matrix (ECM) by ADAMTS5 and presumably by Stromelysin3. One substrate of ADAMTS5 is versican, which is cleaved by this metalloproteinase. In the absence of *Adamts5*, versican is not processed and mice develop syndactyly. However, the fragment of versican processed by ADAMTS5 is able to reestablish cell death in interdigital tissue [46]. There are other components of the ECM that are involved in cell death in the interdigital tissue, such as the α5 chain of Laminin, Fibrillin2, Fibulin1, Nidogen-1, Nidogen-2, and Reelin. Inhibition of cell death in interdigital tissue is observed in *Laminin α5*, *Fibrillin2*, and *Nidogen1* and *Nidogen2* deficient mice or in gene silencing experiments of

the *Reelin* gene or its intracellular effector *Dab1* (Table 2). Additionally, *Fibulin1* is associated with the generation of synpolydactyly in humans [46–50]. Reelin activates the PI3 K-Akt pathway and it is known that Akt phosphorylates the pro-apoptotic molecules BAD, GSK-3β, Procaspase-9, and Forkhead transcription factors, which results in the inhibition of apoptosis [51]. Such cellular interactions between cells and ECM components are important for survival of interdigital tissue.

Table 2. Phenotypes observed in mutant mice for genes related to Extracellular Matrix or in embryonic chick limbs treated with RNAi of *Reelin* or *Dab-1* (see text for details).

Extracellular Matrix	Mutant/Treatment	Model	Phenotype	Reference
	<i>Adamts5</i> ^{-/-}	Mouse	Syndactyly	[46]
	Versican treatment	Mouse	Rescues normal phenotype	[46]
	<i>Fibrillin2</i> ^{-/-}	Mouse	Syndactyly	[47]
	<i>Nidogen1</i> ^{-/-}	Mouse	Syndactyly	[48]
	<i>Nidogen2</i> ^{-/-}	Mouse	Syndactyly	[48]
	<i>Fibulin</i> ^{+/-}	Mouse	Synpolydactyly	[46,50]
	<i>Reelin</i> silencing	Chicken	Promotes cell death	[49,51]
	<i>Dab-1</i> silencing	Chicken	Promotes cell death	[49]

4. Retinoic Acid Signaling

Studies on rodents fed with a Vitamin A-deficient (VAD) diet showed that many organ systems were negatively affected, which reflects the importance of RA during embryonic development [52,53]. RA is derived from retinol (vitamin A) and is obtained from food as carotenoids and retinyl esters. β-carotene is transformed into retinal and then to retinol, whilst the retinyl ester is directly converted into retinol. Retinol binds Retinol Binding Protein 4 (RBP4) and is subsequently transported into the cell by STRA6 and then metabolized into retinaldehyde by retinol dehydrogenases (RDH). In embryonic development, RDH10 is purported to be the most important RDH involved in the metabolism of retinol [54,55]. Additionally, β-carotene can be converted into retinaldehyde by β-carotene oxygenase. Retinaldehyde transforms to RA by action of the retinaldehyde dehydrogenases RALDH1, 2 and 3. Retinoic-acid binding protein II (CRABPII) binds RA and is directed to the nucleus, where CRABPII delivers RA to the RA-receptors (RARs). RARs form heterodimers with retinoid receptors (RXRs) to regulate the transcription of specific genes [53]. The intracellular levels of active RA are regulated by CYP26 (also known as P450RA), a member of the cytochrome P450 family, which converts RA by oxidation into a wide variety of polar metabolites, such as 4-oxo RA and 18-OH-RA [56,57]. The intracellular levels of active RA are under the control of the balance between its synthesis and its degradation.

At advanced stages of limb development, during digit formation, the genes *Rdh10* and *Raldh2* are coexpressed in the interdigital mesenchyme, as well as *Rara* and *Rarb*. Although *Rara* is also expressed ubiquitously during digit formation, *Cyp26b1* is expressed in digit primordia (Figure 2) [58–60]. Also, the Hammertoe mutant mouse has syndactyly in all four limbs because interdigital cell death is inhibited. RA treatment of pregnant Hammertoe females is able to rescue the normal phenotype [61,62]. Studies on *Rdh10* mutant mice demonstrate that in the absence of RA, normal limb patterning occurs, but with syndactyly [59]. Similarly, *Raldh2*^{-/-} rescued mutants at

advanced stages show that RA synthesis is important to control cell death [63]. Regarding the participation of *Rar* and *Rxr* during development, the function of these genes has been evaluated in mutant mice with individual mutations or in combination. Single mutants for any *Rar* or *Rxr* show no effect on interdigital cell death. Double mutants for *Rary*^{-/-} and *Rxrβ*^{-/-} show syndactyly as a consequence of the inhibition of interdigital cell death. Interdigital webbing is also observed in heterodimeric *Rxr/Rar* compound mutants. Conversely, *Rxrα*^{+/-}/*Rarα*^{+/-} and *Rxrα*^{+/-}/*Rary*^{+/-} show mild syndactyly, whereas *Rxrα*^{+/-}/*Rarα*^{+/-}/*Rary*^{+/-} show severe syndactyly indicating that RXRα:RARα and RXRα:RARγ heterodimers are involved in the separation of the digits (Table 3) [64–67].

Figure 2. Expression of *Raldh2*, *Rarβ*, *Cyp26b1* and *Fgf8* in developing chick hind limbs. Whole mount *in situ* hybridization of *Raldh2*, *Rarβ*, *Cyp26b1* and *Fgf8*. (A) *Raldh2* expression pattern at stages 27–29HH and 31HH. (B) *Rarβ* expression pattern at stages 27–30HH. (C) *Cyp26b1* expression pattern at developing stages 26–29HH. (D) *Fgf8* expression pattern at stages 27–29HH and 32HH. Note that expression of *Raldh2* and *Rarβ* is observed in the interdigital tissue, which is down-regulated at stages 30–31HH. *Cyp26b1* is expressed in digital rays. *Fgf8* is down-regulated in the apical ectodermal ridge (AER), corresponding to developing stages in which cell death is observed in the interdigital tissue (compare with Figure 1). Also note that *Raldh2* is expressed in the tendons (arrows) in developing stages 27–29HH. At 31HH, expression is still present in the tendons (arrows), but disappears from the interdigits when cell death begins.

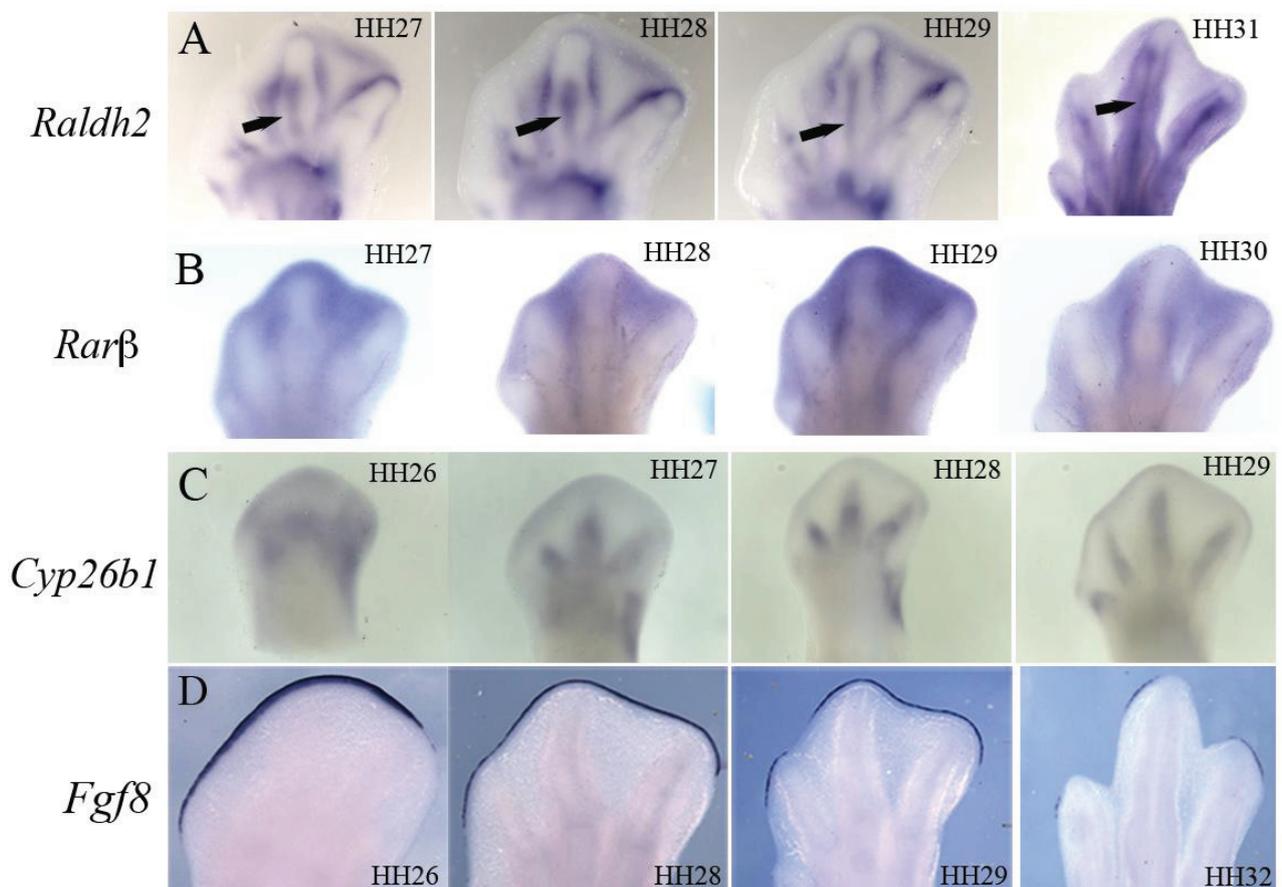
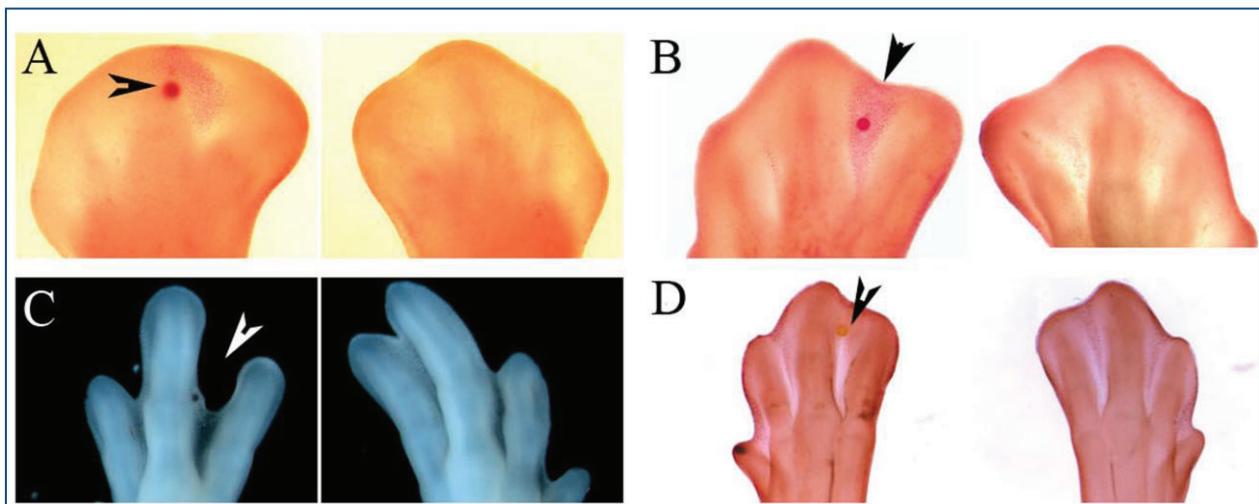


Figure 3. Retinoic acid (RA) participates in interdigital cell death. **(A)** A 50 mM RA-soaked bead was placed in the undifferentiated zone in the tip of the digit at stage 27HH and induced cell death (arrowhead) after 24 h; cell death was not observed in the contralateral limb. **(B)** A 50 mM RA-soaked bead was placed in the interdigital tissue at stage 28HH and induced cell death (arrowhead) after 24 h. Note a wider region of cell death in comparison with the contralateral limb. **(C)** A 50 mM RA-soaked bead was placed in the interdigital zone at stage 29HH and after 48 h accelerated the regression of the interdigital tissue (arrowhead) compared with the contralateral limb. **(D)** A 20 mM AGN193109 (RARs antagonist)-soaked bead was placed in the interdigital region at stage 30HH and after 24 h of treatment interdigital cell death (arrowhead) was inhibited. Neutral red staining is shown in A, B and D.



Remarkably, in chick embryos, the inhibition of RA signaling by the use of a pan-RAR antagonist leads to the inhibition of interdigital cell death and the induction of an ectopic digit (Figures 3 and 5) [68]. In contrast, in mutant mice mentioned above no formation of extra-digit is achieved, just interdigital webbing is seen. The mutant mouse model for synpolydactyly has a mutation in the homeotic gene *HoxD13*, resulting in duplication of one or more digits and the fusion of two or more digits [69]. *HoxD13* is expressed during autopod development and regulates the expression of *Raldh2*; in this mutant the levels of this enzyme are diminished in interdigital tissue, as well as RA production and expression of RA downstream targets genes, such as *RARβ*. Although this mutant has been classified as an animal with polydactyly, extra cartilage formation does not represent true ectopic digits, but rather represents uncontrolled chondrocyte differentiation in the interdigital tissue. The intrauterine treatment with RA in these mutants rescues the normal phenotype [69]. Altogether the results from *Rdh10*, *Raldh2*, *Rar*, *Rxr* mutant mice suggest that RA is important in promoting cell death, in chick embryos and synpolydactyly mutant mice. RA also inhibits chondrogenesis in addition to promoting cell death (Table 3).

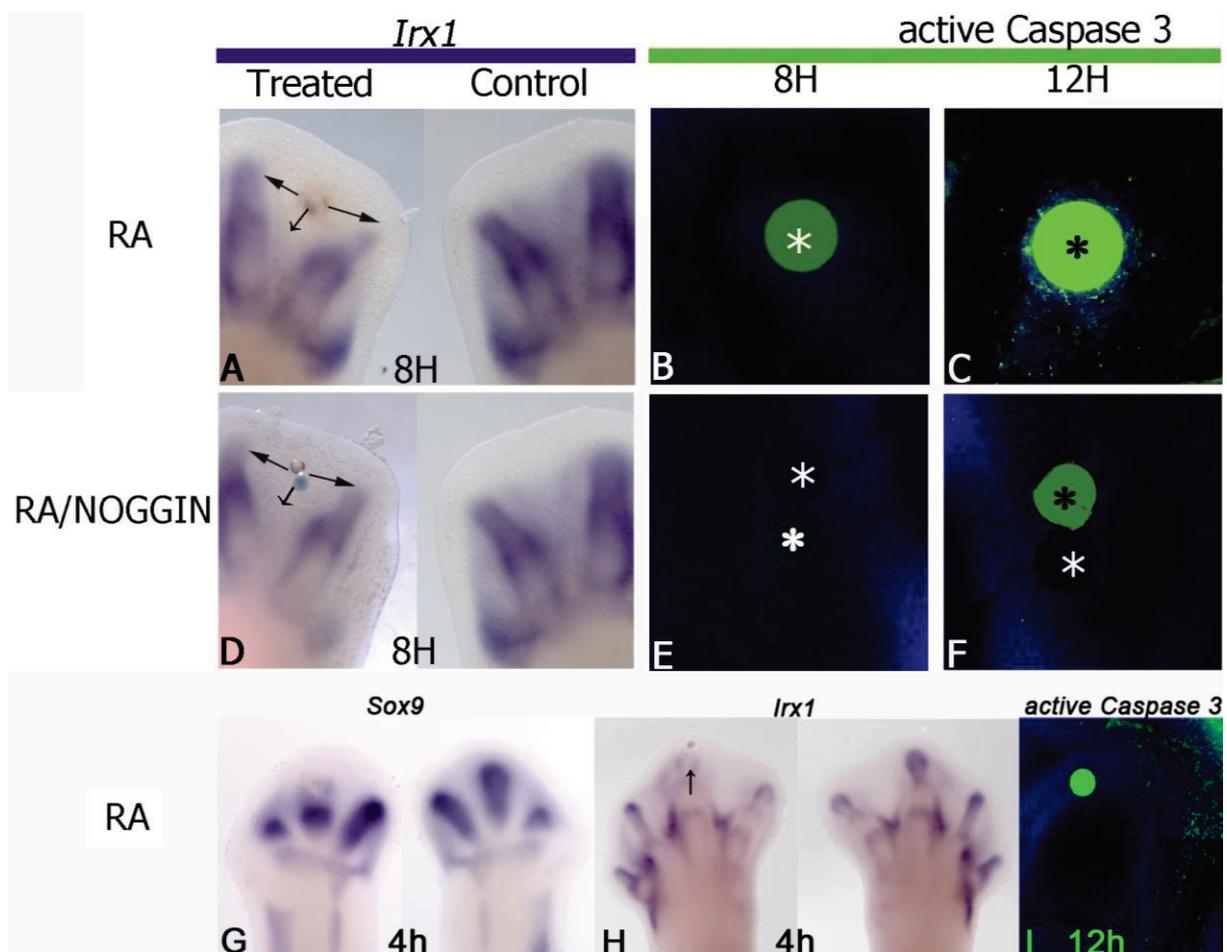
Table 3. Phenotypes observed in mutant mice for genes related to retinoic acid (RA) signaling or in embryonic chick limbs treated with RA or pan-RAR antagonist (see text for details).

	Mutant/Treatment	Model	Phenotype	Reference
RA signaling	Hammertoe mutant treated with RA	Mouse	Rescues normal phenotype	[52]
	<i>Rdh10</i> ^{-/-}	Mouse	Syndactyly	[49]
	<i>Raldh2</i> ^{-/-}	Mouse	Syndactyly	[54]
	<i>Rarg</i> ^{-/-} <i>Rxrβ</i> ^{-/-}	Mouse	Syndactyly	[57,58]
	<i>Rxra</i> ^{+/-} <i>Rara</i> ^{+/-}	Mouse	Mild syndactyly	[57,58]
	<i>Rxra</i> ^{+/-} <i>Rarg</i> ^{+/-}	Mouse	Mild syndactyly	[57,58]
	<i>Rxra</i> ^{+/-} <i>Rara</i> ^{+/-} <i>Rarg</i> ^{+/-}	Mouse	Severe syndactyly	[57,58]
	<i>HoxD13</i> ^{-/-}	Mouse	Synpolydactyly	[60]
	<i>HoxD13</i> ^{-/-} intrauterin treatment with RA	Mouse	Rescues normal phenotype	[60]
	pan-RAR antagonist	Chicken	Inhibition of interdigital cell death	[59]
	Interdigital treatment with RA	Mouse/Chicken	Promotes cell death	[29,59,70]
	<i>Fused toes</i> ^{+/-}	Mouse	Massive cell death	[71]

5. Antagonism between RA and FGF

Although RA is an important factor that potentially induces cell death, there are other factors that work in coordination with it to promote apoptosis. It was mentioned earlier that cells from the undifferentiated zone beneath the AER receive signals that direct them to differentiate or to die. The presence of the AER is important for controlling cell death, it releases the survival factor FGF8 that protects the mesenchyme from cell death [2–4,72]. It is known that the activity of BMP is necessary to inhibit *Fgf8* expression in the AER and to promote cell death [73]. In contrast, activating FGF signaling in the interdigital tissue inhibits cell death, as occurs with the inactivation of BMP signaling (under these conditions expression of *Fgf8* is not inhibited in the AER) [70,74,75]. Interestingly, cooperative functions between BMP and FGF signaling are observed when FGF signaling is inhibited in interdigital tissue and under these conditions BMPs cannot promote cell death [75]. Expression of BMPs depends on RA and expression of *Bmp2*, *Bmp4*, *Bmp5* and *Bmp7* is observed in interdigital tissue, whereas blocking of RA activity inhibits *Bmp* expression [68]. In contrast, FGF8 is able to antagonize RA signaling, since FGF8 inhibits expression of *Raldh2* and induces *Cy26b1* expression [76]. However, RA is also able to antagonize FGF signaling by inhibiting *Fgfr1* expression [76]. Besides, as mentioned above, RA induces *Bmp* expression and BMP inhibits *Fgf8* expression [68,73]. It has been suggested that down-regulation of *Fgf8* in the AER could be the first event that triggers cell death [76].

Figure 4. RA inhibits *Irx* gene expression before the onset of cell death by a BMP-independent mechanism. (A–F) *Irx1* expression is down-regulated in the interdigital area at stage 27HH in chicken hind limbs after 8 hours of RA-treatment and RA/NOGGIN-double treatment. Note that the BMP antagonist NOGGIN does not inhibit the effect exerted by RA on *Irx1* expression. (B,C) Active caspase 3 (green) is analyzed in the interdigital region of hind limbs after RA treatment for 8 h (B) and 12 h (C). (C) The first signs of cell death induced by RA were observed at 12 h. Active caspase 3 was inhibited after RA/NOGGIN-double treatment (E-F), but *Irx1* gene expression remained unaffected (D), indicating that RA inhibits *Irx* expression by a BMP-independent mechanism. (G–I) RA inhibits *Irx1* at the digit tip before the induction of cell death. *Sox9* (G) and *Irx1* (H) expression in hind limbs at stage 27HH after 4 h of treatment with RA, and the presence of active caspase 3 (I) at 12 h after the same treatment in the digit tip. Note that down-regulation of *Sox9* and *Irx1* expression begins before the presence of active caspase 3. In all *in situ* hybridizations, the experimental samples (A, D, G, H) are presented on the left, whereas controls are on the right. Black arrows in A and D indicate the area of *Irx1* inhibition. Black arrows in G and H indicate the bead position. Autofluorescence of ionic-exchange beads used for RA-treatment (black asterisks) is observed as green color. The natural red color of Cy3, used to detect active caspase 3, was changed to green for better visualization of the images. (White asterisks indicate beads soaked in NOGGIN). Modified from [29].



There is another group of genes regulated by RA during digit development: the *Iroquois* genes (*Irx*), which encode homeoproteins that belong to the TALE superclass of homeobox transcription factors with an *Iroquois* box that corresponds to an atypical homeodomain in the protein [71]. In vertebrates, their genomic organization is in two cognate clusters of three genes each; cluster “A” includes *Irx1*, *Irx2*, and *Irx4* and cluster “B”, *Irx3*, *Irx5*, and *Irx6* [71]. It has been observed that during limb development in chick embryos, *Irx1* and *Irx2* belonging to cluster “A” are coordinately expressed in the interdigital tissue and are down regulated by RA; remarkably by a BMP-independent mechanism. *Irx* expression is down-regulated before the observation of first signs of active caspase 3 (Figure 4) [29]. In this sense, it is possible to speculate that IRX1 and IRX2 might play a protective role against cell death. Thus, their down-regulation by RA may be a prerequisite to promote the molecular cascade that ends in cell death. In other models, IRX proteins repress the expression of *Bmp4* or *Msx*; two genes involved in cell death during limb development [71,77–83]. Accordingly, the mutant mouse *fused toes* (characterized by deletion of six genes, which include cluster B of the *Irx* genes) exhibits massive cell death concomitant with up-regulation of *Bmp4* and *Dkk1*, and down-regulation of *Fgf8* and *Fgf10* [71].

6. Antagonism between RA and TGFβ

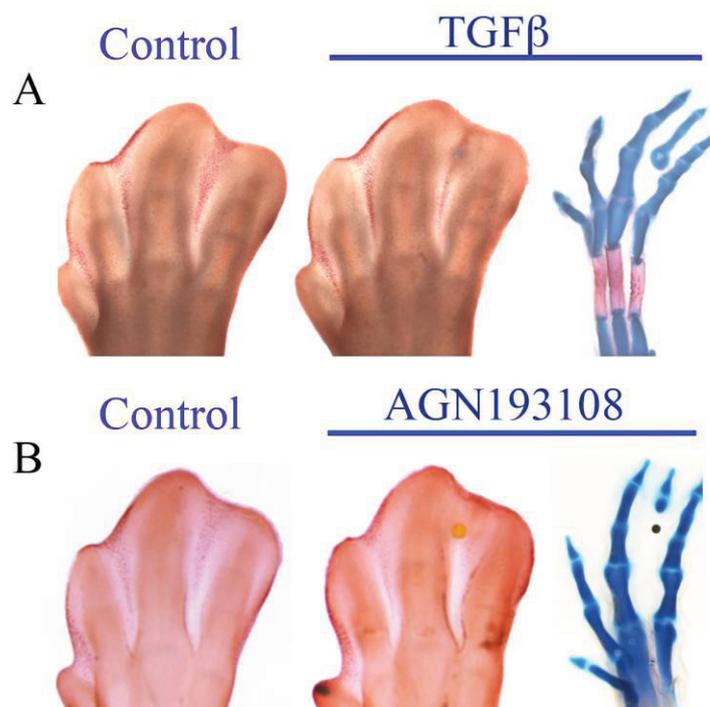
On the basis that blocking the function of RA in interdigital tissue of chick embryos results in ectopic digit formation [27,68], it has been suggested that RA may promote cell death and inhibit cartilage differentiation (Figure 5) [21]. *Sox9* is the master gene for chondrogenesis and is induced as early as 30 minutes after a stimulus of TGFβ/Activin signaling in interdigital tissue [23]. Subsequently, a molecular cascade begins that culminates in the formation of an ectopic digit and inhibition of cell death (Figure 5) [21,84]. During autopod formation, *Sox9* expression is observed in presumptive digital rays and presumptive interdigital tissue [84]. In contrast, when *Sox9* is deleted from undifferentiated mesenchymal cells of limb buds in a conditionally mutant mouse, the skeletal elements do not develop in the autopod, but instead massive cell death is observed in the regions in which digits would have been developed [85].

Interestingly, in the synpolydactyly mutant mouse model, *Raldh2* is down-regulated, which results in the reduction of RA levels and duplication of one or more digits and the fusion of two or more digits [69]. The intrauterine treatment with RA of these animals rescues the normal phenotype [69]. On this basis it is possible to speculate that RA inhibits the recruitment of mesenchymal cells to the chondrogenic lineage, hence becoming cells responsive to the cell death program, whereas cells committed to chondrogenesis in digital rays that express *Cyp26b1* become unresponsive to the cell death program, because CYP26b1 regulates active RA levels, promoting oxidation of RA to polar metabolites [63].

On the other hand, it is interesting to note that *Irx1* and *Irx2* are also expressed in the boundary of skeletal primordia and non-cartilage tissue. RA inhibits these genes, whereas TGFβ has an inductive effect [29]. RA treatment at the tips of digits induces cell death, but before the commencement of cell death, *Irx1* and *Irx2* expression is inhibited and *Sox9* expression is down-regulated (Figures 4 and 6) [29]. In conclusion, the expression of *Irx* genes in response to the antagonism between TGFβ and RA might delimit the boundaries of skeletal elements [29]. Recently, it was shown that RA might

participate to define the digit–interdigit junction, where tissue remodeling occurs, by induction of *Hmgn1* and *Fgf18* expression at the digit–interdigit junction [63]. Interestingly, in another work, it was suggested that *Irx* expression in the boundary of skeletal primordia and non-cartilage tissue (digit–interdigit junction) may reflect the range of diffusion of TGF β to promote chondrogenesis, and IRX proteins may repress the expression of genes involved in the cell death process, hence possibly allowing the formation of the prospective perichondrium [29].

Figure 5. Activation of transforming growth factor beta (TGF β) signaling or inhibition of RAR inhibits interdigital cell death and induces the formation of an ectopic digit. (A) TGF β or (B) pan-RAR antagonist (AGN193108) inhibits cell death and induces the formation of an ectopic digit after 4 days of treatment. The inhibition of RA signaling with a pan-RAR antagonist (AGN193108) gives rise to the same phenotype as treatment with TGF β . For both treatments neutral red staining indicates interdigital cell death. The image of the ectopic digit after TGF β treatment was taken from [29], doi:10.1371/journal.pone.0058549.g003.



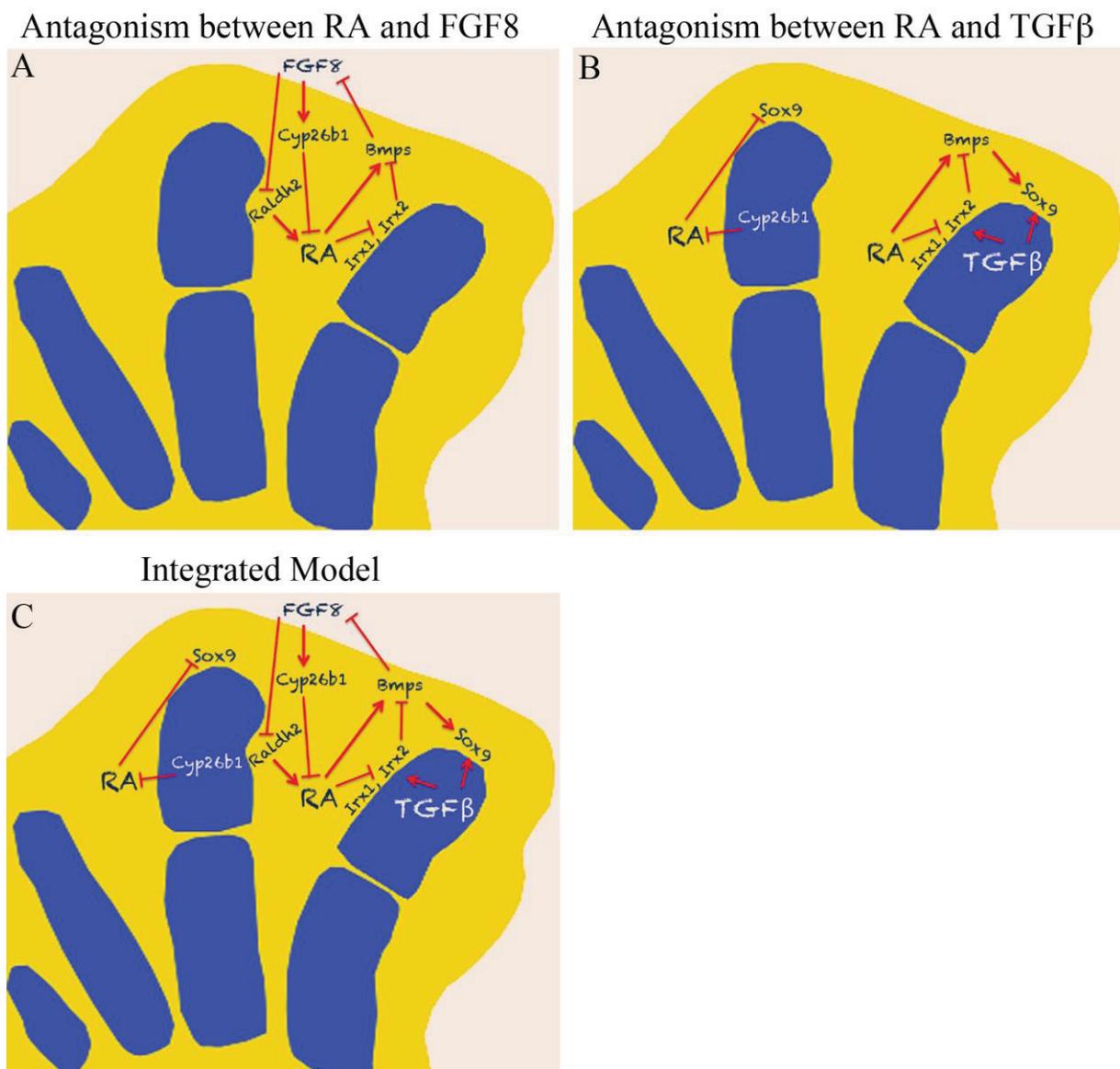
7. Concluding Remarks

The antagonism between FGF and RA signaling plays a crucial role in controlling interdigital cell death and outgrowth of the limb. Likewise, the antagonism between RA and TGF β /Activin signaling in the embryonic chick limb controls cell differentiation during digit formation.

RA promotes cell death by inducing the expression of BMPs, which inhibit *Fgf8* expression (Figure 6A). In addition, RA inhibits the expression of *Irx1* and *Irx2* genes in interdigital tissue. Interestingly, inhibition of *Irx* genes is not mediated by BMPs (Figure 6A). However, it is known that *Irx* genes inhibit the expression of *Msx* and *Bmp4* genes during embryonic development of *Drosophila* and *Xenopus* [71,77–83], suggesting that IRX in the interdigital tissue might have a protective function

against the role of BMPs on promoting cell death [29]. On the other hand, the mutant mouse *fused toes* (characterized by deletion of six genes, which include cluster B of the *Irx* genes) exhibits massive cell death simultaneously with up-regulation of *Bmp4* and *Dkk1*, and down-regulation of *Fgf8* and *Fgf10* [83]. It is reasonable to speculate that RA is required to inhibit *Irx* genes to allow BMPs to carry out their effect in the molecular cascade that culminates in the onset of cell death. In other systems, the deletion of *Irx* genes gives rise to promotion of cell death [80,81]. Also, RA may promote the expression of the pro-apoptotic factor *Bax*, as well as *tissue-transglutaminase* and *stromelysin 3* genes and inhibit BAG-1, a protein with the ability to bind to the anti-apoptotic protein BCL2 [63,76,86,87]. Overall it is possible to speculate that RA may orchestrate the expression of several genes whose proteic products are necessary to be activated or inhibited once the molecular cascade of cell death is activated (Figure 6A).

Figure 6. Schematic representation of antagonism between (A) RA and FGF8 and antagonism between (B) RA and TGFβ. (C) Represents the integrated model with both antagonistic interactions (see the text for details).



Inhibition of *Fgf8* depends on BMPs and, because RA induces expression of *Bmp2*, *Bmp4*, and *Bmp7*, it has been suggested that the antagonism between RA and FGF signaling may be responsible for distal interdigital cell death (Figure 6A) [76]. In mouse embryonic limbs, distal interdigital cell death depends on down-regulation of *Fgf8* in the AER by action of RA [76]. Furthermore, FGF8 promotes cell survival probably by inducing the expression of *Cyp26b1* in the undifferentiated mesenchyme underneath the AER and distal ectoderm and by inhibiting *Raldh2* expression, thereby protecting interdigital tissue from the actions of RA in the induction of the interdigital cell death cascade (Figure 6A) [76].

Regarding chondrocyte differentiation, RA inhibits *Sox9* and, in consequence, chondrogenesis [29,68], probably by preventing the cellular recruitment to the chondrocyte lineage (Figure 6B). Once inhibition of *Sox9* expression occurs, *Irx* genes at the boundary of skeletal elements are inhibited and subsequently cell death occurs [29]. TGF β promotes expression of *Irx* genes at the boundary of skeletal elements, and on this basis, it is possible to speculate that *Irx* gene expression in these regions protects cells committed to chondrocytic lineage against the signals that promote interdigital cell death (Figure 6B) [29]. Also, *Cyp26b1* is expressed at developing digits and probably this enzyme protects cartilage tissue from the actions of RA (Figure 6B) [63].

In conclusion, RA is a molecule that interacts with other signaling pathways in an orchestrated manner to regulate the spatial-temporal expression of several genes involved in the activation of cell death or inhibition of cell differentiation (Figure 6C).

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Author Contributions

MED-H, RFA-B and JC-M have written about digit development and programmed cell death in the limb mesoderm. MED-H, AJR-F, MB and JC-M have written about retinoic acid signaling and antagonisms between RA and FGF and between RA and TGF β . All authors participated in the design of figures and tables.

Conflicts of Interest

The authors declare no conflict of interest.

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