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UNUSUAL MODEL SYSTEMS FOR CELL DEATH RESEARCH

Sculpturing digit shape by cell death

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Abstract Physiological cell death is a key mechanism that ensures appropriate development and maintenance of tissues and organs in multicellular organisms. Most structures in the vertebrate embryo exhibit defined areas of cell death at precise stages of development. In this regard the areas of interdigital cell death during limb development provide a paradigmatic model of massive cell death with an evident morphogenetic role in digit morphogenesis. Physiological cell death has been proposed to occur by apoptosis, cellular phenomena genetically controlled to orchestrate cell suicide following two main pathways, cytochrome C liberation from the mitochondria or activation of death receptors. Such pathways converge in the activation of cysteine proteases known as caspases, which execute the cell death program, leading to typical morphologic changes within the cell, termed apoptosis. According to these findings it would be expected that caspases loss of function experiments could cause inhibition of interdigital cell death promoting syndactyly phenotypes. A syndactyly phenotype is characterized by absence of digit freeing during development that, when caused by absence of interdigital cell death, is accompanied by the persistence of an interdigital membrane. However this situation has not been reported in any of the KO mice or chicken loss of function experiments ever performed. Moreover histological analysis of dying cells within the interdigit reveals the synchronic occurrence of different types of cell death. All these findings are indicative of caspase alternative and/or complementary mechanisms responsible for physiological interdigital cell death. Characterization of alternative cell death pathways is required to explain vertebrate morphogenesis. Today there is great interest in cell death via autophagy, which could substitute or act synergistically to the apoptotic pathway. Here we discuss what is known about physiological cell death in the developing interdigital tissue of vertebrate embryos, paying special attention to the avian species.

Keywords Interdigital cell death · Caspases · Cathepsins · BMP · FGF · Retinoic acid

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Regulation and significance of interdigital cell death during the formation of the digits in vertebrate embryos

Programmed cell death is a basic mechanism employed in embryonic systems to sculpt the morphology and the structure of the developing organs (morphogenetic and histogenetic cell death) or to remove organs or tissues with transitory functions in the embryo (phylogenetic cell death; see [1]). The developing vertebrate limb is one of the best characterized paradigms where programmed cell death has a central role in morphogenesis [2]. The embryonic limb is a simple structure consisting of a core of mesodermal cells covered by an ectodermal jacket, which bulges as a bud in the lateral surface of the embryonic body (Fig. 1a). The mesodermal cells of the early limb bud have skeletogenic potential but are maintained undifferentiated and proliferating by the influence of a thickened region of the ectoderm

occupying the distal margin of the bud, termed the Apical Ectodermal Ridge (AER; see Fig. 1b–e). The AER produces fibroblast growth factors (FGFs), which act on the subjacent mesodermal cells that constitute the Progress Zone (PZ). FGF8 is the main FGF of the AER, but FGF4, FGF9, FGF17 and FGF19 are also present, during development. Intense proliferation of the mesodermal cells of the distal region of the bud under the influence of the AER is accompanied by progressive differentiation at more proximal regions of the limb bud (Fig. 1f–g). This process forms the chondrogenic primordia of the limb in a characteristic proximal–distal sequence appearing first the primordium of the femur/humerus (stylopod), next those of the tibia-fibula/ulna-

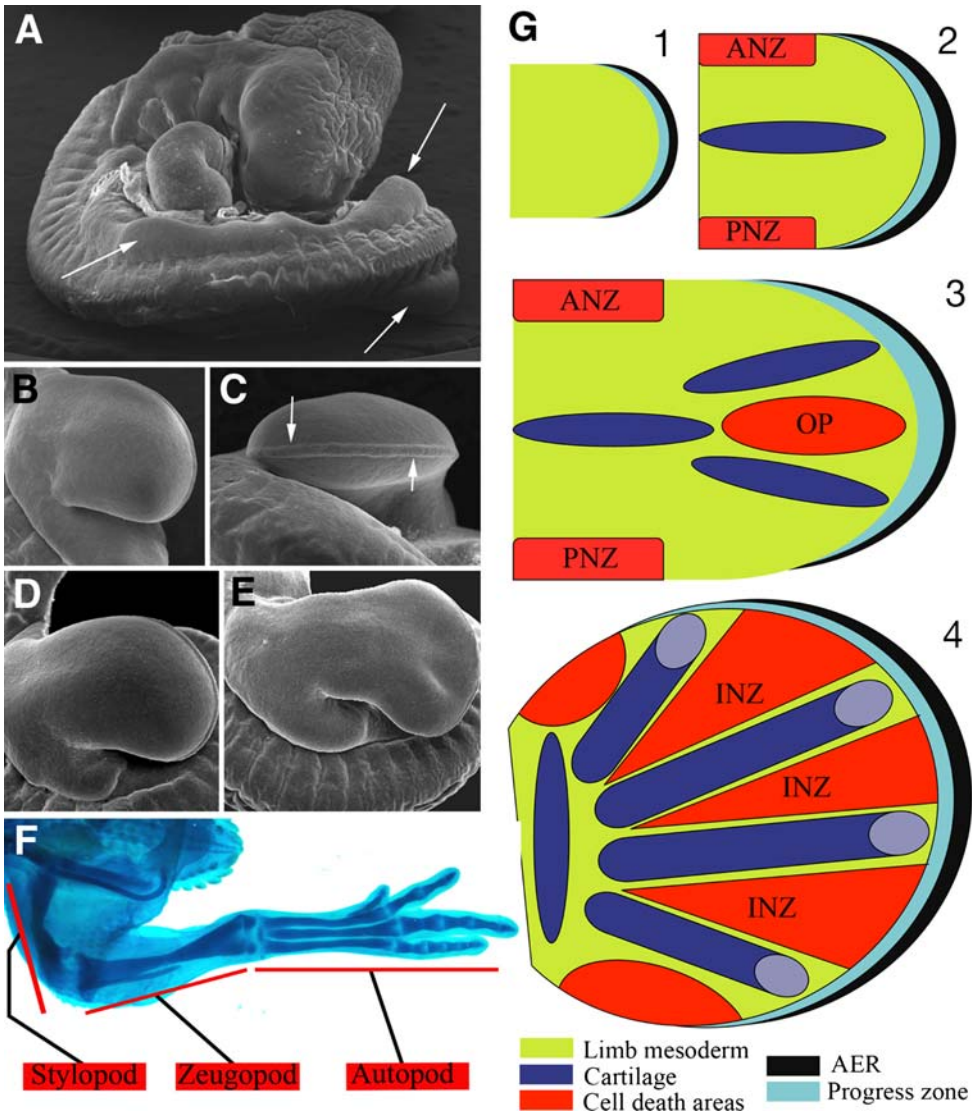

Fig. 1 Limb development and cell death areas. **a–e** Scanning electron micrographs that illustrate the early stages of limb development in a chicken embryo. Limbs form as small buds that emerge from the flanks of the developing vertebrate embryo (*Arrows* in **a**). Limb buds are simple structures consisting of a mass of mesodermal cells covered by an ectodermal jacket. As development takes place limb buds growth along the three axis (**b–e**). Early on, at the distal edge of the limb, a local thickening of the ectoderm forms the apical ectodermal ridge (*AER*), a key structure responsible of limb outgrowth (*arrow* in the frontal view of the limb bud in **c**). **f** illustrates on a chicken developing limb, the three regions that become specified along the proximal–distal axis within the limb, the Stylopod, Zeugopod and Autopod. **g** Schematic drawings representing limb bud development in order to illustrate the areas of cell death. As limbs growth, different areas of cell death appear concomitantly to skeletal elements differentiation. The initial undifferentiated mass of mesodermal cell contributes to limb bud growth as they proliferate within the Progress Zone (*PZ*) of the limb (*1*), which is influenced by the inductive signals from the apical ectodermal ridge (AER) (*1*). As differentiation of skeletal elements of the Stylopod (*2*) and the Zeugopod (*3*) starts in the core of the mesodermal tissue, the remaining mesoderm begin to be removed by cell death, appearing the Anterior Necrotic Zone (*ANZ*), the Posterior Necrotic Zone (*PNZ*) and the Opaque Patch (*OP*). In the same fashion within the autopod (*4*), as digits differentiate, the interdigital mesoderm is removed by cell death in the Interdigital Necrotic Zones (*INZs*)

radius (zeugopod) and finally the carpal/tarsal pieces and the digits (autopod) (Fig. 1f).

A remarkable feature is that, the mesodermal cells of the bud, which do not integrate into the developing cartilages, undergo apoptosis forming well defined regions of cell death around these chondrogenic aggregates (Fig. 1g). Among these areas of cell death, the most remarkable are those located between the developing digits, which have been initially termed the interdigital necrotic zones (INZs), as they were described when the term apoptosis was not yet established [3–5]. Other regions of cell death, such as the anterior and posterior necrotic zones (ANZ and PNZ) or the opaque patch (OP) are located in the peripheral margins of the limb bud, and between the zeugopodial cartilages [3–5]. Additionally cell death is also associated with the establishment of the muscle bellies, which develop from a distinct mesodermal cell population invading the limb from the adjacent somites [6]. Apoptosis is also present in the core of the prechondrogenic aggregates establishing the zone of joints formation [7].

All these areas of cell death are finely regulated and play a variety of roles including sculpturing the gross morphology of the limb [8–10] and the shape of the muscle bellies and tendons [6], and to establish the zones of joint formation [7]. The formerly termed interdigital necrotic zones (INZs) are regions of massive cell death, which remove the undifferentiated mesodermal cells located between the developing digits and cause the freeing of the digits from the hand or foot plate (see Fig. 2).

Morphogenetic role of INZ: interdigital tissue regression versus differential growth

INZ's are present in most tetrapod embryos but their extent varies among species, exhibiting in most cases a close relationship with the final morphology of the digits. In the chick embryo, interdigital cell death is part of a complex process of tissue regression, which lasts more than 2 days. At the beginning of the process, interdigital mesenchymal cells die by apoptosis. However this is soon accompanied by regression of interdigital blood vessels [11] and disintegration of the extracellular matrix scaffold of the interdigit, which result in detachment of tissue fragments into the amniotic sac [12]. In birds with webbed digits, as the duck, INZ's are rudimentary in comparison with that of the chick but the sequence of degenerative events is similar between both species [13]. Reptiles show a pattern of interdigital cell death similar to that of birds including differences in death intensity between species with free digits (i.e. lizards) and species with webbed digits (i.e. turtle) [14].

It has been proposed that the morphogenetic role of INZ′s in mouse embryos is less relevant than in birds [15, 16]. According to this interpretation there is no proper interdigital tissue degeneration in the mouse. Instead, cell death accompanies a differential growth process of the autopod characterized by intense growth of the digit tips and negative growth of the interdigital regions. However as in birds and reptilians, the morphology of the digits in

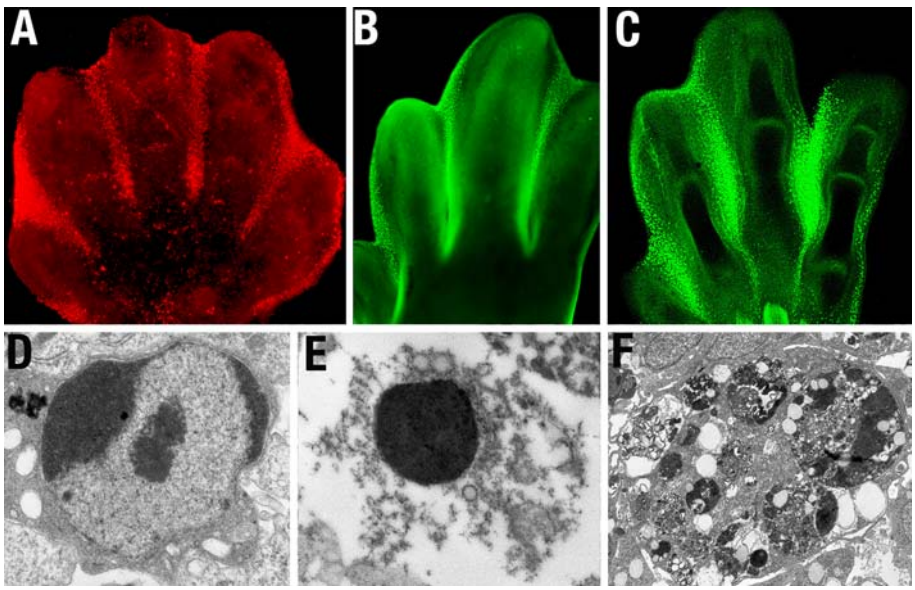

Fig. 2 Interdigital Cell Death in the Developing Limb. **a–c** Images in **a** and **b** shows cell death occurring in the interdigital tissue of a mouse (**a**) and a chicken embryo manifested by TUNEL labeling (**a**) and Acridine Orange staining (**b**), respectively. Image in **c** shows the high levels of Cathepsin expression in the regressing interdigital tissue as manifested by Cathepsin D immunolabeling. **d-f** Electron microscopy images showing cell types that are found in the regressing avian interdigital tissue. **d** illustrates a typical apoptotic cell, **e** shows a characteristic necrotic cell while in **f** we can appreciate a macrophage

mammals is closely associated with the pattern and the intensity of interdigital cell death. In this regard interdigital cell death is dramatically reduced or absent in the bat, which is a specie with a webbed digits phenotype [17]. In any case the presence of both free digits species (mice, chicken, lizard, etc.) and webbed digits species (bat, duck, turtle, etc.) within each class of vertebrate, provides researchers with potentially extraordinary tools to study the morphogenetic role of cell death during development [18].

Interdigital cell death is a characteristic feature of amniote embryos. In amphibians digits form largely by selective outgrowth of the digit tips in total absence of INZ [19, 20]. However, it has been reported that some amphibian species exhibit areas of interdigital cell death resembling that reptilian, avian or mammals [21].

Cytological features of INZ: loss of cell–matrix adhesion, apoptosis, cell fragmentation and phagocytosis

Cell death in the interdigital mesoderm follows a morphological pattern of apoptosis [7, 22]. Transmission electron microscopy studies have shown that most interdigital dying cells appear rounded and electron dense with the nucleus exhibiting a characteristic peripheral condensation of the chromatin (Fig. 2d). The apoptotic cells undergo fragmentation and the resulting debris is removed by phagocytosis. Removal is performed both by the neighboring healthy cells and by incoming macrophages of hematopoietic origin (Fig. 2f). Studies in PU.1 null mouse embryos indicate that local mesenchymal cells are able to substitute for professional phagocytes, and indeed interdigital tissue regression in these macrophageless mice follows almost the same schedule as in wild type animals [23].

Regardless of the predominant apoptotic morphology of the dying interdigital mesenchymal cells, it is remarkable that at peak stages of degeneration cells bearing morphological features of necrosis are relatively abundant in chick interdigits [24] (Fig. 2e). This fact may be interpreted as the coexistence of apoptotic and necrotic pathways or that apoptotic cells undergo secondary necrosis if phagocytic removal is delayed (see below for more details).

Interdigital mesenchyme isolated from the developing limb up to 12 h prior to the onset of INZ establishment exhibits considerable chondrogenic potential when cultured at high density [22]. This indicates that irreversible commitment to dye does not take place until a few hours prior to death. Immunocytological analysis of interdigital cells during the time period preceding overt apoptosis, combining a panel of nuclear and cytoplasmic markers with TUNEL labeling, allowed the identification of the earliest cell alterations associated with cell death [25]. At the nuclear level, degradation of acetylated histone 4 and alteration in the components of the nuclear membrane (lamin B and nucleoporin) accompanied by anomalies in the nucleo-cytoplasmic traffic are precocious degenerative events [25]. In the cytoplasm microtubules and actin microfilaments appeared conserved at initial stages of apoptosis, while vimentin intermediate filaments show clear signs of disorganization and degradation. The maintenance of N-CAM immunolabeling in TUNEL positive cells suggests that cell–cell adhesion is relatively conserved in dying cells. In this regard, interdigital mesenchyme bears specific domains of protocadherin gene expression [26]. However, alterations in components responsible for cell matrix adhesion, such as paxillin, were early events in the degenerative process [25]. Furthermore, the interdigital extracellular matrix scaffold is particularly complex in the stages preceding INZ [27] and undergoes intense remodeling by a variety of metalloproteinases specifically up-regulated in the regressing interdigits (Stromelysin-3 [28]; Adamts5 [29, 30]). On the basis of these findings it was proposed that the INZ might constitute an example of cell death associated with the loss of cell–matrix adhesion, often termed anoikis [25]. This interpretation is reinforced by the occurrence of syndactyly in mouse mutants with alterations in a variety of matrix components [30–35]. However, it cannot be forgotten that interdigital matrix may serve also a function in storing and targeting secreted growth factors implicated in the control of the INZ [36] (see also below).

Cell death machinery in the INZ: mitochondrial permeabilization, ROS, caspases and lysosomes

The apoptotic machinery responsible for cell death in the INZ has not yet been fully elucidated. The occurrence of soft tissue syndactyly in mice lacking both Bax and Bak function [37] and in mice lacking Bax and the BH3-only protein Bim [38] indicates that these pro-apoptotic members of the bcl-2 family play a critical role in the induction of INZ. Moreover, all these factors are functionally associated with the *intrinsic apoptotic pathway* associated with permeabilization of the outer mitochondrial membrane. Hence, it can be assumed that the mitochondria is the first target for the interdigital cell death triggering signals (see below). Consistent with a central role of mitochondrial dysfunction in the INZ, at peak stages of interdigital cell death cytochrome c is released into the cytosol and the mitochondrial apoptotic inducing factor AIF is translocated into the nucleus of cells in the regressing interdigital mesenchyme [25]. Furthermore, the interdigital antiapoptotic factor Bag-1 (Bcl-2 associated athanogene 1), which

interacts and co-localizes in the mitochondria with BCL-2, is expressed in the interdigital mesoderm of the mouse limb at stages preceding apoptosis, becoming progressively down-regulated in correlation with the progress of the degenerative process [39].

The death effectors of the INZ acting downstream of mitochondrial permeabilization remain more elusive. The absence of an interdigital phenotype in mice with different genetic modifications to functionally abolish a large number of, if not all, potential candidate effectors is indicative of intense redundancy in these factors. In the canonical intrinsic apoptotic pathway, mitochondrial permeabilization is followed by cytosolic release of cytochrome c, which activates caspase 9 upon binding to the adaptor protein Apaf-1 and procaspase 9 to form the *apoptosome*. While there is evidence for the activation of caspase 9 in the INZ [40], syndactyly is not observed in knock out mice for either Apaf-1 [41] or Caspase 9 [42]. However, in Apaf1$^{-/-}$ mice interdigital cell death is delayed and acquires morphological features of necrosis [41], suggesting the activation of alternative cell death mediators to executor caspases in absence of apoptosome activation.

In the canonical mitochondrial apoptotic pathway, caspase 9 activates the executioner caspases 3, 6 and 7 (see [43]). As for caspase 9, there is evidence for the involvement of these executioner caspases in INZ [25, 44], but no interdigital phenotype is induced in mice deficient in either caspase 3 [45], caspase 6 or caspase 7 [46]. Together these findings indicate the existence of alternative cell death pathways able to substitute for the canonical apoptotic pathway mediated by caspases.

Caspase 2 is highly expressed by dying interdigital cells [25] and could be a good candidate to substitute for other apoptotic mediators following blockade of the intrinsic apoptotic pathway. In some systems caspase 2 is able to promote mitochondrial permeabilization [47], and can also activate caspase 3 [48]. However, silencing interdigital expression of caspase 2 with siRNA delays but do not inhibits cell death in the INZ [25]. Furthermore, syndactyly is not induced in mice deficient in this caspase [49].

In sum, all the above mentioned data indicates that apoptotic caspases acting downstream of mitochondrial permeabilization are active in INZ but they can not be the sole apoptotic factors. In this regard treatments with the pan-inhibitor of caspases Z-VAD-FMK only reduce but not inhibit cell death in the INZ [50].

There is evidence showing that formation of reactive oxygen species (ROS) and the release of lysosomal enzymes are also mechanisms accounting for cell death in the INZ. ROS are toxic compounds generated in the cells as consequence of aerobic metabolism. In physiological conditions ROS activity is modulated by antioxidant factors, and cell death and several degenerative diseases, including aging, can be caused by deficiencies in the capability of cells to neutralize these compounds [51]. At the stages of active cell death, the interdigital tissues exhibit high levels of reactive oxygen species (ROS) and, most important, treatments with antioxidants can reduce cell death in the INZ [52–54]. However, it is unlikely that ROS exert their influence in the INZ directly, as knock out mice deficient for enzymes responsible for neutralizing ROS are viable and lack an abnormal phenotype [54]. It is more likely that ROS appear as a consequence of other cell death pathways in regulating cell death in the INZ. Thus, the local interdigital increase in ROS might be due to the mitochondrial permeabilization secondary to Bax activation [55].

Lysosomes have been largely considered responsible for non-apoptotic cell death and phagocytic elimination of dead cells. However, there is increasing evidence for the involvement of lysosomes in death processes in which cell morphology corresponds with apoptosis. It has been proposed the term “apoptosis-like programmed cell death” to define this type of cell death [56]. Furthermore, analysis of cell death after treatment with lysosomotropic detergents revealed that the lysosomal induction of apoptotic versus necrotic features might be explained by differences in the intensity of lysosomal permeability [57, 58]. The role of lysosomes in cell death in the INZ is supported by the following facts: (1) lysosomal cathepsins genes (*cathepsin B*, *D* and *L*) are up-regulated in the non-macrophagic interdigital mesenchyme during the stages of interdigital tisssue regression; (2) the occurrence of lysosomal permeabilization in interdigital mesenchymal cells during interdigital tissue regression; (3) the induction of apoptosis in primary cultures of undifferentiated embryonic limb mesenchyme overexpressing *cathepsin D*; and, (4) by the increased reduction of cell death in the INZ in chick autopods subjected to combined treatments with caspase and cathepsin D inhbitors, in comparison with interdigits treated only with caspase inhibitors [50].

The mechanism responsible for lysosomal permeabilization in the INZ has not been yet analyzed. In some systems Bax activation has been shown to be associated with lysosomal permeabilization [59]. Moreover ROS are important factors able to induce lysosomal permeabilization [60, 61]. Hence, both mechanisms might reflect a crosstalk between different cell death pathways ensuring the elimination of the interdigital mesenchyme. In addition, lysosomal enzymes delivered into the cytosol might also induce mitochondrial permeabilization [62] and activation of the executioner caspases [63] forming an interactive loop able to reinforce the initial death signal. In this regard, it must be mentioned the absence of interdigital phenotype both in mice lacking different key members of the caspase-mediated death pathways (see above) and also in mice

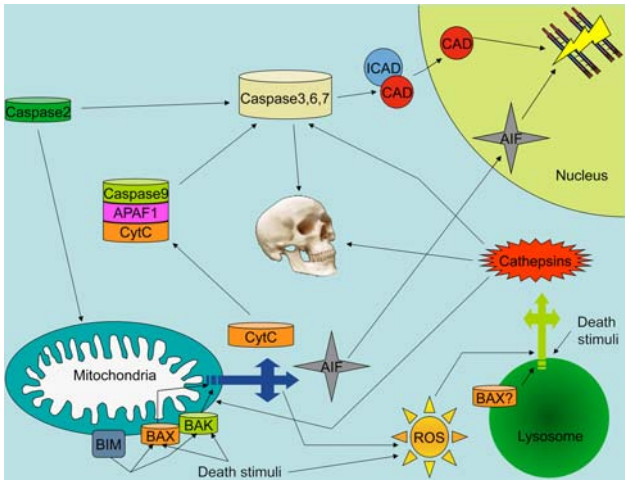

Fig. 3 Interdigital Cell Death Pathway. The scheme illustrates the mitochondria and the lysosome as independent interconnected partners playing central roles in the cell death cascade of the interdigital cells. CytC: Cytochrome C; AIF: Apoptotic Inducing Factor; ICAD: Inhibitor of Caspase Activated DNase; CAD: Caspase Activated DNase; ROS: Reactive Oxigen Species; APAF1: Apoptosis Protease-Activated Factor 1

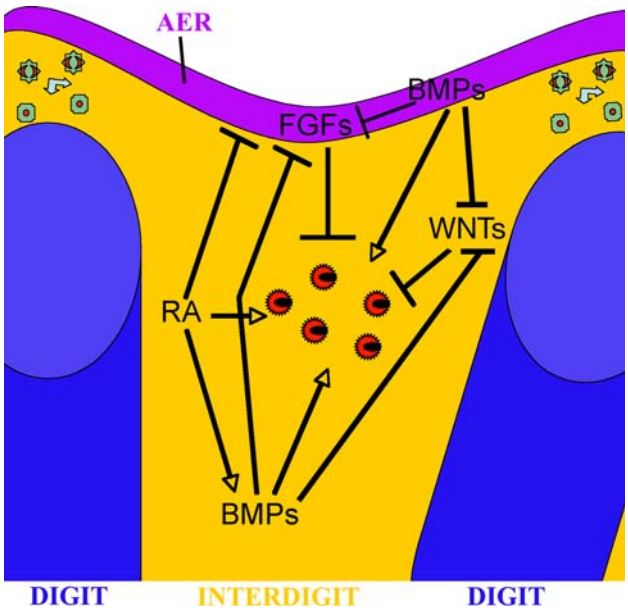

Fig. 4 Regulators of Interdigital Cell Death. This figure shows a schematic drawing of the interdigital tissue with the flanking forming digits where we represent the main factors involved in the stimulation of cell death. FGFs: Fibroblast Growth Factors; RA: Retinoic Acid; BMPs: Bone Morphogenetic Proteins;WNTs: Wnt factors; AER: Apical Ectodermal Ridge

deficient in different cathepsin genes [64–66]. Fig. 3 illustrates a schematic representation of the interaction of different dying pathways accounting for cell death in the INZ.

Molecular regulation of INZ: FGF, BMP and RA signaling

The hand/foot develop from the distal portion of the limb bud, which is termed the autopod. In this region the interdigits occupy the space delimited between two digit primordia. Each interdigit has a structure quite similar to the early limb bud, as they are formed by a core of undifferentiated mesodermal tissue, covered by ectoderm with a distinctive AER along their distal margin (see Fig. 4). As in the early limb bud, the tissue components of the interdigit establish complex interactions through the production of a variety of secreted signaling molecules that control cell fate and tissue differentiation. Among these secreted factors, Fibroblast Growth Factors (FGFs), Bone Morphogenetic Proteins (BMPs) and Retinoic acid metabolites (RA) have been identified as signals responsible for the regulation of cell fates in the INZ. However, their precise role and interactions remain uncertain.

FGF signaling

The AER produces FGF8 which maintains the subjacent mesoderm undifferentiated and proliferating accounting for limb outgrowth. The onset of cell death in the INZ coincides temporarily with the physiological cessation of AER function, which is accompanied by down-regulation of *Fgf8* gene expression [18]. In concordance with a role for this secreted factor in the control of cell death, INZ formation is inhibited after local application of exogenous FGFs at cell death stages [67]. Furthermore, syndactyly is observed in the human syndromes caused by a constitutive activation of FGF receptors [68] and mice lacking *Fgf4,8,9* and *17* genes in the AER have rudimentary limbs that exhibit ectopic regions of mesodermal cell death during development [69–71]. Together these findings are consistent with a role for FGFs as survival signals for undifferentiated limb mesoderm. However, there is also evidence showing that FGFs are required for cell death. Hence, local inhibition of FGF signaling causes syndactyly in chicks [72]. In addition, initial inhibition of interdigital cell death induced by local application of FGFs into the chick interdigit, is followed by a dramatic intensification of cell death 2 days after the treatment, when the source of exogenous FGFs loss its activity [72]. Consistent with this finding, local application of FGFs into the interdigits of the webbed digits of duck embryos [72] increases the sensitivity of the duck interdigital mesenchyme to undergo cell death by treatments with BMPs (see below).

The apparently contradictory effects of FGFs have been interpreted as evidence for a dual role of FGFs as a survival signal which, at the same time, sensitizes the limb mesoderm to the effect of the apoptotic triggering signals [72]. It

is likely that apoptotic sensitivity of the undifferentiated mesenchymal cells is related to the regulation of N-Myc expression by FGFs [73].

BMP signaling

The family of Bone Morphogenetic Proteins (BMPs) includes cytokines that play crucial roles in limb development, thus their members are involved not only in early patterning [74–76] but also in cartilage formation [75, 77, 78], joint specification [79] or apoptotic cell death induction [77]. It has been suggested that different BMP receptors and crosstalk with different signaling molecules might be involved in the different responses to BMPs in the limb autopod [80, 81].

Numerous experiments performed in chick and mouse embryos point to BMPs as responsible molecules for the onset of cell death in the INZ. Genetic approaches in mice indicate that the apoptotic promoting effect of BMPs is secondary to the inhibition of *Fgf* gene expression in the AER [16, 70] but it cannot be discarded a direct cell-autonomous proapoptotic effect of BMPs on the mesodermal cells.

In the regressing interdigits there are two sources of BMPs, the AER and the mesoderm [82, 83]. The AER express *Bmp2*, *Bmp4* and *Bmp7* [74, 83, 84]. At initial stages of limb development these expression domains contribute to establish the dorso-ventral axis of the limb bud [74, 75], but during the stages of digit formation they appear to be implicated in the regression of the AER [18, 74, 83], which is followed by cell death in the subjacent mesoderm. Hence it has been proposed that ectodermal BMPs are central players in the control of INZ cell death by regulating the expression of *Fgf8* in the AER [70, 74].

The interdigital mesoderm shows also prominent expression domains of *Bmp2*, *Bmp 4*, *Bmp5* and, *Bmp7* genes [84, 85] and gain-of-function experiments by local application of beads bearing any of those BMP proteins into the interdigital mesenchyme, prior to establishment of the INZ or in the early limb bud, induces massive cell death by apoptosis [77]. In a complementary fashion, loss-of-function experiments to block BMP signaling in the interdigits inhibit cell death and cause syndactyly [83, 86–89]. From these experiments it is difficult to establish whether the control of cell death in the INZ depends on the ectodermal or the mesodermal BMPs or even both, as local treatments with BMPs in the chick limb induces a precocious regression of the AER [18]. However, consistent with a role of mesodermal BMPs in the control of INZ formation, the functional activity of the mesodermal BMPs is finely tuned by two BMP antagonists, BAMBI [85, 90] and inhibitory-smads [91] expressed in the interdigits. Furthermore, the webbed interdigits of duck embryos express *Bmp* genes in a pattern similar to those of the chick, but their expression is accompanied by well defined expression domains of the secreted BMP antagonist *Gremlin* [86]. This feature is observed also in the webbed wing of bat embryos [17].

RA signaling

Retinoic acid (RA) is the representative name of a set of active metabolites of vitamin A produced in the embryo by the enzymatic action of Raldh2. RA signals through a variety of nuclear receptors, which are ligand-inducible transcriptional regulators. RA is indispensable for embryonic development (see [92]) and plays important roles in the developing limb [93, 94]. During the formation of the digits, all the components of this signaling pathway including *Raldh2*; the retinoic acid receptors beta and gamma (*RARbeta*; *RARgamma*) and *Cyp26b1*, which encodes an enzyme responsible for the inactivation of retinoic acid, are all expressed in association with the developing digits. Of these genes, *Raldh2* and *RARbeta* are specifically expressed in the interdigital mesoderm [95, 96]. In chick embryos local interdigital treatments with all-trans-RA induce premature cell death and regression of the AER, while local application of a RA antagonist inhibits interdigital cell death leading to syndactyly [97]. Mesenchymal cell death is also observed in mice treated with RA or genetically modified to silence *Cyp26b1* [98, 99] and mice deficient in both RARbeta and RAR gamma exhibit soft tissue syndactyly [28].

Whether RA regulates cell death in the INZ directly or indirectly remains unclear. RA signaling up-regulates *Bmp* gene expression in the interdigital mesoderm [28, 97], and interdigital apoptosis induced by RA is attenuated or inhibited by combined treatment of RA with a BMP antagonist [97]. In addition, RA might also influence cell death through neutralizing the survival effect of FGF8 [16, 94] or inhibiting differentiation of interdigital mesoderm into cartilage [96]. Together these findings are suggestive of an indirect effect of RA on formation of the INZ. Consistent with this interpretation, interdigital expression of *Bax* which is a key regulator of INZ formation (see above) is not differentially regulated in RA loss-of-function experimental approaches [28] (but see also [16]).

Notch and Wnt signaling

While FGFs, BMPs and RA appear to be major regulators of INZ formation, other signaling pathways might also be implicated in this process as deduced by specific expression patterns in the regressing interdigits and/or by the occurrence of syndactyly when the pathway is genetically disturbed. One of the candidates is the Notch pathway

signaling, which in fact is modulating apoptotic cell death in the developing limb at the level of the AER [100]. The formation and maintenance of the AER is one of the basic roles of the Notch pathway during limb development where it controls cell death modulating the level of *Fgf8* expressing cells in the AER [100]. Consistent with this statement, mice deficient in the Notch ligands, *Serrate* or *Jagged2*, display syndactyly after showing expanded expression domains of *Fgf8* and a considerable reduction on the expression of *Bmps* in the interdigital tissue [101–103].

Members of the Wnt signaling pathway, including the ligand *Wnt5a* and the receptor *Fz4*, are expressed in the regressing interdigital tissue [104] but a possible role in the control of cell death in the INZ still awaits clarification. Wnt factors might be considered survival signals during limb development. In this regard it has been suggested that Thalidomide, which is known to cause massive apoptotic cell death during limb development, may exert its teratogenic effects by the inhibition of WNT signaling after stimulation of ROS production [105]. In support for a role of WNT signaling in limb cell survival in physiological conditions, is the finding that one of the ways for BMPs to induce apoptotic cell death works through stimulation of Dickkopf1 (Dkk1), a WNT signaling inhibitor [106]. Thus, mice deficient in *Dkk1*, which is highly expressed in the regressing interdigital tissue, show syndactyly phenotype [107, 108]. Syndactyly is also found in mice mutants for other potential antagonist of WNT pathway as *Megf7* [109] or *Sfrp2* [110] supporting the antiapoptotic role for WNT signaling in the interdigital tissue.

Concluding remarks

The areas of interdigital cell death constitute one of the best illustrative models showing the implication of programmed cell death in embryonic morphogenesis. It is remarkable that at least in birds, this physiological degenerative process involves not only cell death but also tissue regression, including collapse of blood flow and tissue disintegration. This feature must cause important homeostatic modifications able to influence the functional properties of the enzymatic cascades implicated in the cell death mechanisms. In this regard, the changes in milieu pH conditions are particularly important, since they can drastically modulate not only the function of proteins but also their subcellular locations (see [111]). Hence it is not surprising that this degenerative process implicates multiple intracellular degenerative pathways, involving caspases, mitochondria, lysosomes and ROS. A mayor task for future research in the area is to unravel the molecular coordination and interactions between these different degenerative machineries.

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