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Caspase inhibition supports proper gene expression in *ex vivo* mouse limb cultures

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Abstract

We standardized conditions for *ex vivo* mouse limb culture to study cartilage maturation and joint formation. We compared 12.5 d.p.c. mouse forelimbs that were cultured either mounted or freely rotating for up to 72 h. Limb outgrowth progressed *ex vivo* at a variable rate as compared to its development *in vivo*, spanning approximately 48 h. Although cartilage maturation and joint formation developed grossly normal, aberrant expression of skeletal marker genes was seen. Interestingly, no regression of the interdigital webs took place in mounted cultures, in contrast to limited webbing under freely rotating conditions. Caspase inhibition, by addition of zVAD-fmk to the culture medium of freely rotating limbs, supported proper gene expression associated with skeletal development, and prevented interdigital web regression. Taken together, a freely rotating *ex vivo* culture for mouse limb outgrowth that is combined with caspase inhibition provides a good model to study cartilage maturation and joint formation. *Cell Death and Differentiation* (2001) **8,** 985–994.

Keywords: mouse limb outgrowth; cartilage maturation; joint formation; interdigital web regression; caspase inhibition

Abbreviations: *abc1, ATP binding cassette transporter-1*; ALP, alkaline phosphatase; BMP, bone morphogenetic protein; *c1qa, complement component 1qa*; caspase, cysteine aspartic acid specific protease; *col2a1, collagen type 2a1*; d.p.c., days post coitum; *gdf5, growth and differentiation factor-5*; TGF-β, transforming growth factor-β; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling; zVAD-fmk, benzyl-oxycarbonyl-Val-Ala-Asp(O-methyl)-fluoromethylketone

Introduction

At midgestation of mouse embryonic development, limb outgrowth[1] provides an interesting model to study cartilage maturation, joint formation and webbing. Interdigital web regression, a prominent morphogenetic event, is the ultimate result of apoptosis of the interdigital tissues. For a long period, webbing has only been connected to the shaping of the limbs.[2] However, recently it has been demonstrated that interdigital tissues also establish digit identification,[3] prior to their regression. Simultaneously, mesenchymal cells condense inside the outgrowing limb to form the cartilaginous templates of the skeleton.[4] Interestingly, while mesenchymal condensations lie down as full rays, the resulting matured cartilaginous elements are interrupted at specific sites by future joint interzones.[5,6] The rapid expansion of molecular approaches in the field of developmental biology, including *in situ* hybridization, allowed identification of signaling pathways associated with these morphogenetic events. Morphogens involved in these processes include members of the Transforming Growth Factor-β/Bone Morphogenetic Protein (TGF-β/BMP) and Wnt superfamilies, as well as retinoic acid.[5–11]

Data revealing the expression patterns of genes that are possibly involved in skeletal and joint development have to be expanded with functional studies, ultimately defining their biological relevance. Up to date, loss-of-function studies have been performed *via* gene targeting (knock-outs, knock-ins). Conventional knock-outs are often embryonically lethal even before the onset of skeletal development, or can yield a phenotype that masks the latter process. Therefore it may be desirable to use conditional knock-outs with tissue specific promoter elements. However, gene targeting remains elaborative, expensive and time consuming, which hampers its routine use. Overexpression models (transgenes) address gain-of-function: we successfully generated transgene animals carrying the gene encoding *cartilage-derived morphogenetic protein-1/growth and differentiation factor-5 (gdf5)* controlled by the *collagen type 2a1 (col2a1) or col11a2* promoters,[12] allowing overexpression of the gene specifically in cartilage. So far, tissue specific promoter elements that allow gene targeting/overexpression in diarthrodal joints are still lacking. One can anticipate that promoter elements[13] of joint specific markers such as *gdf5*[14] can drive expression of exogenous genes in the joint interzone.

In avian species, functional studies can be conveniently carried out using *in ovo* manipulation of the embryonic limb: for instance beads soaked in morphogens can be implanted, or the limb can be infected[15] using retrovirus. Such a manipulated embryo can further be incubated and grown under physiological conditions until analysis of the skeletal phenotype. Despite the practical advantages associated with *in ovo* manipulation of avian embryos, it remains necessary to study mammalian limb outgrowth, since the molecular cascades involved in skeletal and joint development differ in part between these animal classes.[16–19] In the past decades, developmental biologists and teratologists already made sporadic attempts to apply *ex vivo* mouse limb outgrowth to perform functional

studies.[20–24] However, this model has never been well standardized. Although the data obtained could be interpreted conclusively at the histological level, the validation of the model using molecular techniques such as *in situ* hybridization appears to be limited.[24] Recently, *ex utero* surgical procedures for mouse limb manipulation have also been reported.[25,26] However, it is a very tedious and technically demanding procedure not readily suitable for routine use to date.

Here we report on the standardization of the culture conditions for *ex vivo* mouse limb outgrowth. Our data reveal that cartilage maturation and joint formation can develop grossly normal in explanted limbs, independently of interdigital web regression. We furthermore demonstrate that caspase inhibition supports proper expression of skeletal marker genes, as analyzed by *in situ* hybridization. The described model allows future functional studies.

Results

Standardization of culture conditions for *ex vivo* mouse limb outgrowth

To develop standardized serum free culture conditions for *ex vivo* mouse limb outgrowth, we compared the two most commonly used setups. Therefore, 12.5 days post coitum (d.p.c.) mouse forelimbs were incubated either mounted or freely rotating (Figure 1). The limbs were allowed to further develop for up to 72 h. Interestingly, inspection of the general morphology of limbs in mounted cultures indicated the absence of interdigital web regression (Figure 1, upper panel) in contrast to limited regression using freely rotating conditions (Figure 1, lower panel). As outlined in the forthcoming section, time kinetic analysis revealed that the observed interdigital web regression under freely rotating conditions occurred within 24 h, but did not proceed thereafter (Figures 2, 3 and 5). Supply of additional nutrients such as 10% fetal bovine serum did not significantly improve the culture conditions. Also, preliminary experiments suggested that enhanced oxygen tension (45% O_2)[27] does not significantly improve the outcome of *ex vivo* mouse limb culture, especially with respect to webbing and apoptosis of the ectoderm (data not shown).

Different morphogenetic events progress *ex vivo* at a variable rate as compared to their development *in vivo*

Inspection of the general morphology of explanted limbs already indicated that different morphogenetic events developed differentially during mounted or freely rotating culture conditions. Therefore, we compared cartilage maturation and joint formation in more detail to their development *in vivo*. We examined the maturation of mesenchymal condensations into cartilaginous elements separated by future joint interzones histologically by toluidine blue staining on cultured limbs *versus* limbs collected between 12.5 and 15.5 d.p.c. *in vivo* (Figure 2). In both culture conditions, cartilage maturation and establishment of future joint interzones progressed *ex vivo* to a developmental stage that is comparable to 14.5 d.p.c. *in vivo*,

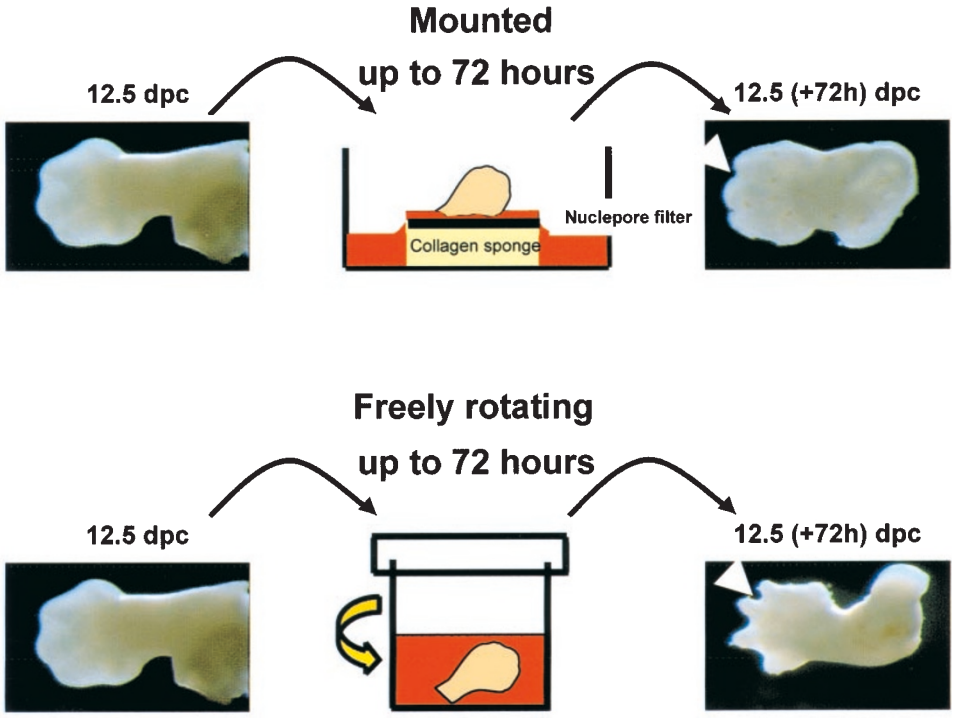

Figure 1 Schematic representation of the conditions used to develop a standardized model for *ex vivo* mouse limb culture. 12.5 d.p.c. mouse forelimbs were cultured either mounted (upper panel) or freely rotating (lower panel). General morphology of representative limbs is shown before (left) and after (right) 72 h of culture. Arrowheads point at interdigital tissues

spanning approximately 48 h. The mesenchymal condensations underwent differentiation into chondrocytes that produced an extracellular matrix composed of sulfated proteoglycans, and moreover gave rise to cartilaginous elements that retained grossly their normal shape.

The maturation of the cartilaginous elements is followed by endochondral ossification completing the developmental process that creates the calcified skeleton.[28] Since the appearance of primary ossification centers within cartilaginous elements can be observed at 14.5 d.p.c. *in vivo*, we next examined if mouse limbs grown *ex vivo* can also undergo endochondral ossification. To detect the early onset of osteogenesis, we visualized histochemically alkaline phosphatase (ALP) activity[29] (Figure 3). As expected, endogenous ALP activity could be detected in the perichondria and in the primary ossification centers of cartilaginous elements of limbs cultured up to 72 h, irrespective of the conditions used. Even more pronounced than in the process of cartilage maturation, the upregulation of endogenous ALP activity followed a proximo-distal pattern. However, the pattern of ALP-positive cells in the perichondria of cultured limbs was often broadened and sometimes extended into the interdigital tissue, in contrast to the sharply delignated ALP-positive perichondria that border cartilaginous elements *in vivo*.

In addition to histological and histochemical analysis, we performed whole mount *in situ* hybridization on explanted limbs to detect the spatio-temporal expression of skeletal marker genes during culture (Figure 4). We chose to analyze *col2a1* expression in chondroblasts and chondrocytes of the cartilaginous skeleton,[30] as well as *gdf5* expression in cells restricted to the narrowing joint interzones.[14] First, the previously described expression patterns of these marker genes were reproduced in limbs collected between 12.5 and 15.5 d.p.c. *in vivo*. In contrast, aberrant gene expression patterns were observed in limbs cultured *ex vivo*. Although *col2a1* expression could still be detected in the cartilaginous elements of the digits and radius/ulna, its expression was almost absent in the carpal/metacarpal region. Furthermore, *gdf5* was expressed barely above the detection limit and the pattern no longer reflected its expression *in vivo*.

Caspase inhibition supports proper expression of skeletal marker genes

Most likely, the failure to detect proper gene expression patterns in explanted limbs could be attributed to cell death of the tissue. Therefore, we performed a terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling (TUNEL) assay to detect nuclear DNA fragmentation in cells that undergo apoptosis[31] (Figure 5). At 12.5 d.p.c. of mouse limb outgrowth *in vivo*, apoptotic cells could be detected in the interdigital tissues and more discrete within future joint interzones. When interdigital web regression proceeds between 13.5 and 15.5 d.p.c., a small number of apoptotic cells remained present underneath the ectoderm, as well as in the joint interzones. In limbs that were cultured *ex vivo*, the presence of TUNEL-positive cells was more pronounced and increased dramatically in time. The staining was no longer restricted to the interdigital tissues and future joint interzones, but was more abundant in a core of soft tissue located at the carpal/metacarpal region of the limb.

Despite the presence of apoptotic cells in the interdigital tissues of limbs that were cultured mounted, webbing did not proceed. This observation prompted us to investigate if clearance of apoptotic bodies was affected using this culture condition. Phagocytosis of cellular debris by

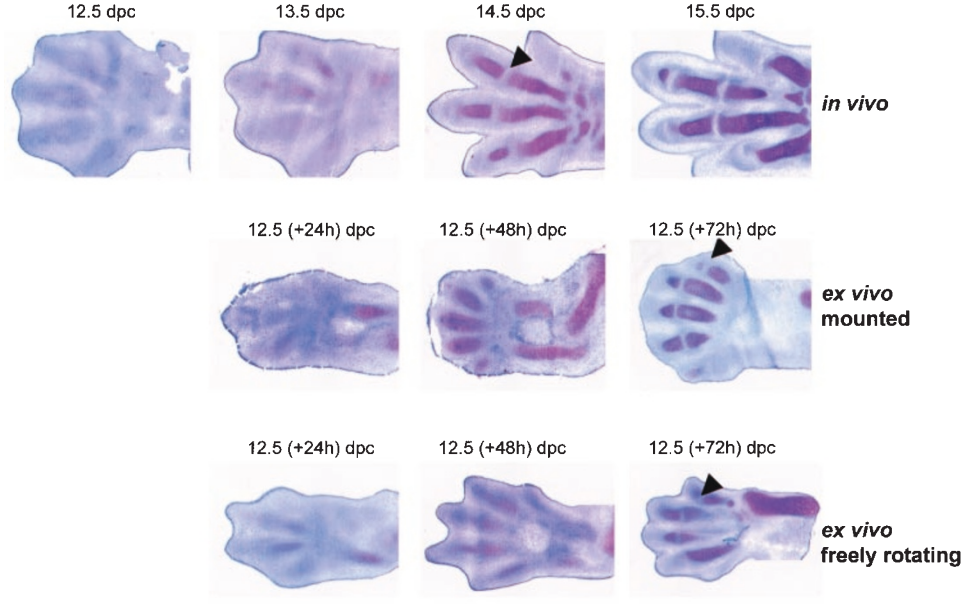

Figure 2 Cartilage maturation and formation of joint interzones during *ex vivo* mouse limb culture as compared to their development *in vivo*. Mouse forelimbs collected between 12.5 and 15.5 d.p.c. *in vivo* or 12.5 d.p.c. mouse forelimbs cultured for 24, 48 and 72 h *ex vivo* using mounted or freely rotating conditions were cryosectioned and stained with toluidine blue. Arrowheads point at joint interzones

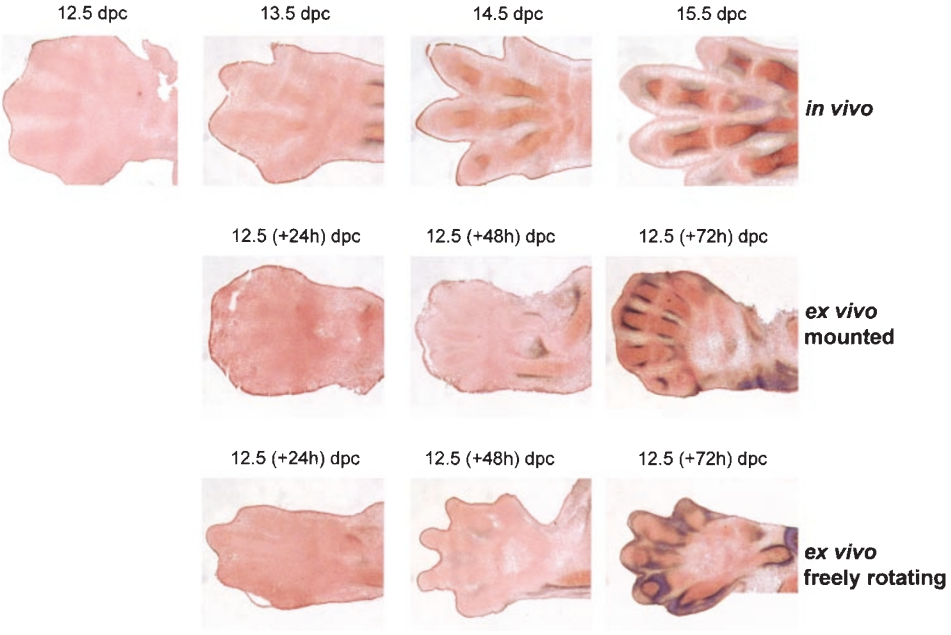

Figure 3 Onset of osteogenesis during *ex vivo* mouse limb culture as compared to its development *in vivo*. Histochemical ALP activity was performed on cryosectioned mouse forelimbs collected between 12.5 and 15.5 d.p.c. *in vivo* or on cryosectioned 12.5 d.p.c. mouse forelimbs cultured for 24, 48 and 72 h *ex vivo* using mounted or freely rotating conditions

macrophages requires expression of various engulfing genes. Therefore, we analyzed by *in situ* hybridization the expression patterns of the molecular marker genes *ATP binding cassette transporter-1 (abc1)*[32,33] and *complement component 1qa (c1qa)*[33,34] in explanted limbs as compared to counterparts *in vivo* (Figure 6). In agreement with the previously described expression patterns, both *abc1* and *c1qa* transcripts were visualized within regressing interdigital tissues of 13.5 d.p.c. mouse forelimbs. In limbs cultured mounted, both genes were also highly expressed in the soft tissue of interdigital webs, resembling the localization of apoptotic bodies as detected by TUNEL assay. These data demonstrate that phagocytosis of apoptotic debris is likely to proceed well during *ex vivo* mouse limb culture, and cannot account for the lack of interdigital web regression using mounted conditions.

To preserve the soft tissue of explanted limbs from apoptosis, we added the cysteine aspartic acid specific protease (caspase)[35] inhibitor benzyloxycarbonyl-Val-Ala-Asp(O-methyl)-fluoromethylketone (zVAD-fmk)[36,37] to the culture medium. Lack of interdigital web regression was observed in limbs that were cultured freely rotating in the presence of 20 μM zVAD-fmk (Figures 4 and 7), in contrast to counterparts to which 0.04% DMSO was added as a vehicle control (data not shown). In agreement, TUNEL-assay performed on zVAD-fmk treated limbs showed a significant decrease in the number of apoptotic cells in the carpal/metacarpal region (Figure 7). When whole mount *in situ* hybridization for *col2a1* and *gdf5* was performed under these conditions, their proper gene expression patterns were observed (Figure 4). Indeed, *col2a1* could now be detected additionally within the carpal/metacarpal region. Furthermore, *gdf5* expression was visualized in a pattern that is restricted to the future joint interzones. In addition, the expression pattern of *gdf5* became sharper delignated within these joint interzones, resembling the developmental stage 14.5 d.p.c. *in vivo*. Similar data, although less pronounced, were obtained using mounted culture conditions in the presence of 20 μM zVAD-fmk (data not shown).

The lack of interdigital web regression in zVAD-fmk treated limbs appeared to be no consequence of cytotoxicity of the inhibitor. Indeed, cartilage maturation, formation of future joint interzones and onset of osteogenesis progressed similarly in treated *versus* non treated counterparts (Figure 7).

Discussion

In the present study, we standardized culture conditions for *ex vivo* mouse limb outgrowth. The conditions require mounted or freely rotated incubation in serum free medium under normal oxygen tension. Supply of additional nutrients such as 10% fetal bovine serum did not significantly improve the culture conditions. Previously published experiments[27] indicated that increase in oxygen tension supports proper maturation of tegumental structures. Our preliminary experiments suggest that enhanced oxygen tension does not significantly improve the outcome of *ex vivo* mouse limb culture, especially with respect to webbing and apoptosis of the ectoderm. At the histological and histochemical level, both culture conditions allowed mesenchymal cell condensations to mature equally well into cartilaginous elements interrupted by future joint interzones. The absence or presence of gentle fluid flow during mounted or freely rotating conditions, did not influence the morphogenetic events studied during the initial phase of skeletal and joint development. These results are in

agreement with former data obtained in paralysed chick embryos,[38–40] showing minor effects of limb immobilization on cartilage maturation and no interference with the formation of future joint interzones. During later stages of chick embryonic development only, absence of joint cavitation was observed using paralyzed conditions, which has been attributed to fusion of opposing cartilaginous elements.

In contrast, interdigital web regression is the most prominent morphogenetic event that is altered during the *ex vivo* culture conditions. A recent study described that interdigital webs are involved in the specification of digit identity, demonstrating an early patterning function for this tissue before its regression.[3] Persistence of the interdigital webs, even throughout complete adulthood, has been described in the natural mouse mutant *Hammertoe*.[41] Despite the interesting observation that this phenotype can be rescued by addition of retinoic-acid,[42] candidate genes disrupted by the mutation have only recently been identified.[43] Furthermore, severe delay of interdigital web regression is encountered as part of the phenotype associated with *Apaf1* knock-out mice.[44,45] *Apaf-1*, the mammalian homolog of *C. elegans* CED-4, has been characterized as an adaptor molecule involved in the activation of specific caspases.[35] Although necrosis-mediated interdigital web regression has also been described under particular experimental conditions,[46] it is generally accepted that interdigital web regression is caused by caspase-mediated apoptosis.[2] Interestingly, small cell permeable tripeptides have been designed that are broad spectrum inhibitors of caspases,[36,37] although non-specific effects on cathepsin inhibition have recently been reported.[47] In agreement with previous reports,[48–50] our observation that addition of such an inhibitor to the medium of explanted limbs blocks interdigital web regres-

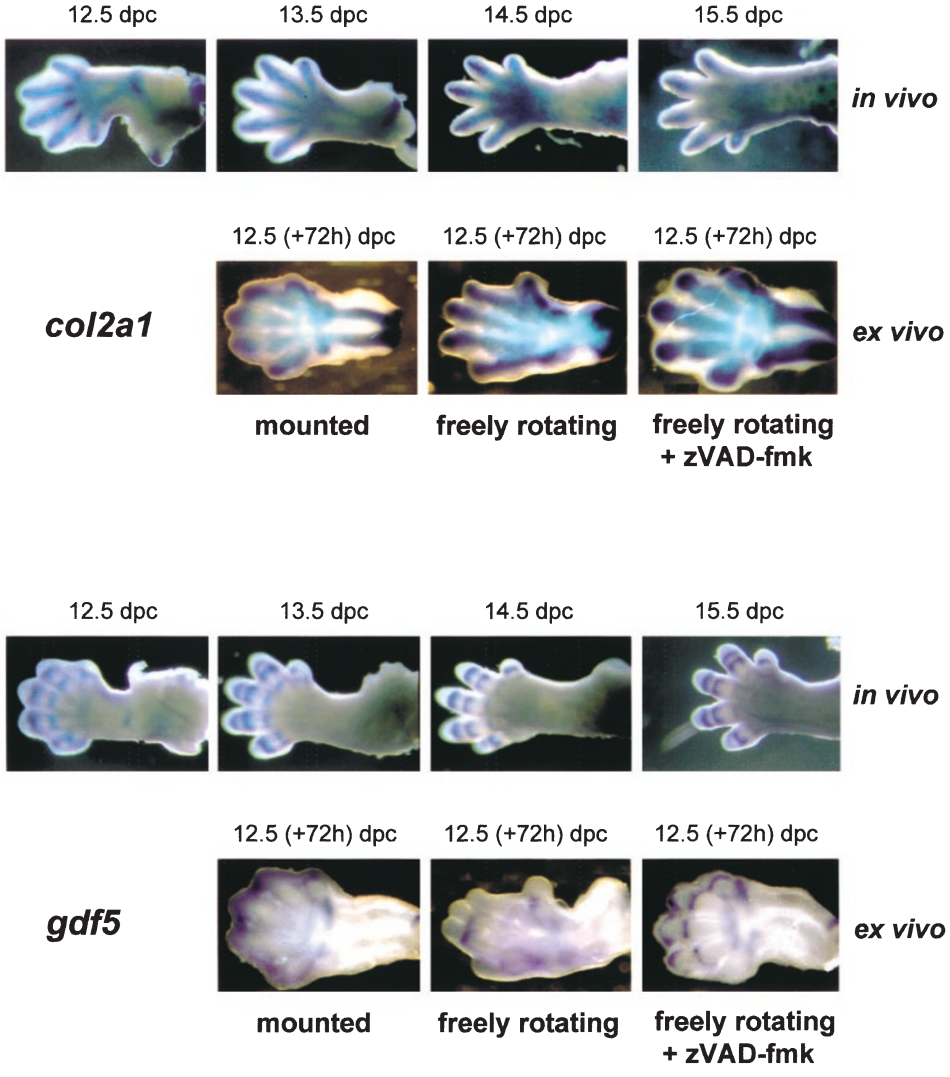

Figure 4 Caspase inhibition supports proper expression of skeletal marker genes in *ex vivo* mouse limb cultures. Whole mount *in situ* hybridization with *col2a1* (upper panel) or *gdf5* (lower panel) probes was performed on mouse forelimbs collected between 12.5 and 15.5 d.p.c. *in vivo* or on 12.5 d.p.c. mouse forelimbs cultured for 72 h *ex vivo* using mounted conditions or freely rotating conditions in the absence or presence of 20 μM zVAD-fmk

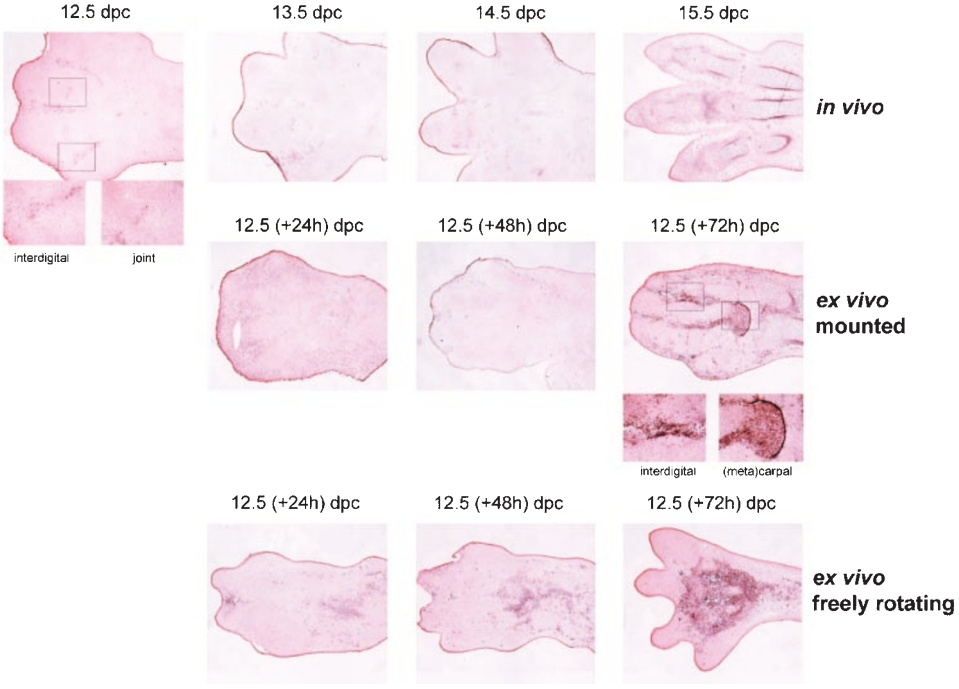

Figure 5 Presence of apoptosis during *ex vivo* mouse limb culture as compared to its detection during development *in vivo*. TUNEL assay was performed on cryosectioned mouse forelimbs collected between 12.5 and 15.5 d.p.c. *in vivo* or on cryosectioned 12.5 d.p.c. mouse forelimbs cultured for 24, 48 and 72 h *ex vivo* using mounted conditions or freely rotating conditions

sion further points to the involvement of caspases in this process. Interestingly, digit individualization in developing limbs in culture can also be prevented by addition of antioxidants, suggesting that generation of reactive oxygen species participates also in the control of embryonic cell death.[51]

At present, the underlying mechanism by which interdigital web regression is completely or partially blocked during mounted or freely rotating culture conditions is unclear. In every respect, the pathway leading to apoptosis proceeds correctly, since even increased numbers of TUNEL-positive cells could be detected within the interdigital tissues of explanted limbs, as compared to counterparts *in vivo*. Also, phagocytosis that allows clearance of cellular debris upon apoptosis appeared not to be affected during *ex vivo* mouse limb culture. Macrophage recruitment to interdigital tissues during limb outgrowth *in vivo*[52] is required for optimal engulfment of apoptotic bodies.[53] Transbilayer redistribution of phosphatidylserine on the membranes of both phagocyte and prey is indispensible for the proper recognition of the cellular corpses, which is at least partly mediated by the activity of the ABC1 transporter protein. Similar to our observations, an increased number of TUNEL-positive particles could be visualized within the interdigital tissues of ABC1-deficient mouse limbs.[54] Furthermore, macrophage-mediated phagocytosis of apoptotic bodies requires lysosomal activity, which may be affected by the culture conditions used. Indeed, a recent study revealed that immunohistochemical detection of the lysosomal membrane glycoprotein LAMP-1 in limbs that were subjected to *ex vivo* culture could be correlated to the degree of digit separation.[55] Interestingly, it was recently described that phagocytosis of apoptotic debris in the developing limb can be taken over by 'stand-in' mesenchymal neighbors in the absence of functional macrophages.[33]

The main advantage of our 'upgraded' model for *ex vivo* mouse limb outgrowth above systems previously used by others, is the correct gene expression pattern analyzed by *in situ* hybridization. This technique is nowadays critical in the field of modern developmental biology and allows interpretation of data at the molecular level. Previous attempts to perform functional studies on skeletal and joint development in explanted mouse limbs have been hampered once solid conclusions had to be made beyond the histological level.[24] Some investigators could circumvent these limitations by dissecting out the separate skeletal elements from the surrounding soft tissue,[56] which excludes however possible biological interactions between different cell types in a physiological environment.

Taken together, the standardization of culture conditions for *ex vivo* mouse limb outgrowth revealed that skeletal and joint development can progress independently of interdigital web regression. The model is further validated by the observation that caspase inhibition supports proper gene expression. Therefore, the presented 'upgraded' model for *ex vivo* mouse limb outgrowth may now be applied as a fast and reliable assay to screen the biological relevance of novel molecules possibly involved in skeletal and joint development, prior to or in conjunction with functional studies using transgene and gene targeting technology.

Materials and Methods

Mouse embryonic limbs

Embryos were collected between stages 12.5 and 15.5 d.p.c. from pregnant CD1 mice. Noon of the presence of a vaginal plug after natural mating was designated as 0.5 d.p.c. The forelimbs were carefully dissected at the level of the elbow joint under a dissection microscope using microscissors.

Ex vivo mouse limb organ culture

Mouse embryonic limbs (12.5 d.p.c.) were cultured for up to 72 h in a humidified atmosphere (37°C, 5% CO_2). Two different culture conditions were used (Figure 1): limbs were mounted in a 24-well chamber on top of a Nuclepore polycarbonate filter (Costar, Badhoevedorp, The Netherlands) placed on a Helistat collagen sponge (Colla Tec, Plainsboro, NJ, USA) or alternatively cultured freely rotating (5 rounds per min, 45° angle) in a bijou bottle (Bibby Sterilin, Staffordshire, UK). Sponges were soaked, or limbs were directly submerged, in 500 μl of BGJ_b medium (Gibco BRL, Merelbeke, Belgium) supplemented with an antibiotic mixture (penicillin (100 units/ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml); Gibco BRL) and bovine serum albumin (1 mg/ml; Serva, Heidelberg, Germany). In comparative studies, submerged limbs were flushed with a gas mixture enriched in oxygen[27] (45% O_2/50% N_2/5% CO_2), or 10% fetal bovine serum (Bio Whittaker, Verviers, Belgium) was added to the culture medium. The tripeptide caspase inhibitor zVAD-fmk was purchased from Biomol (Plymouth Meeting, PA, USA),

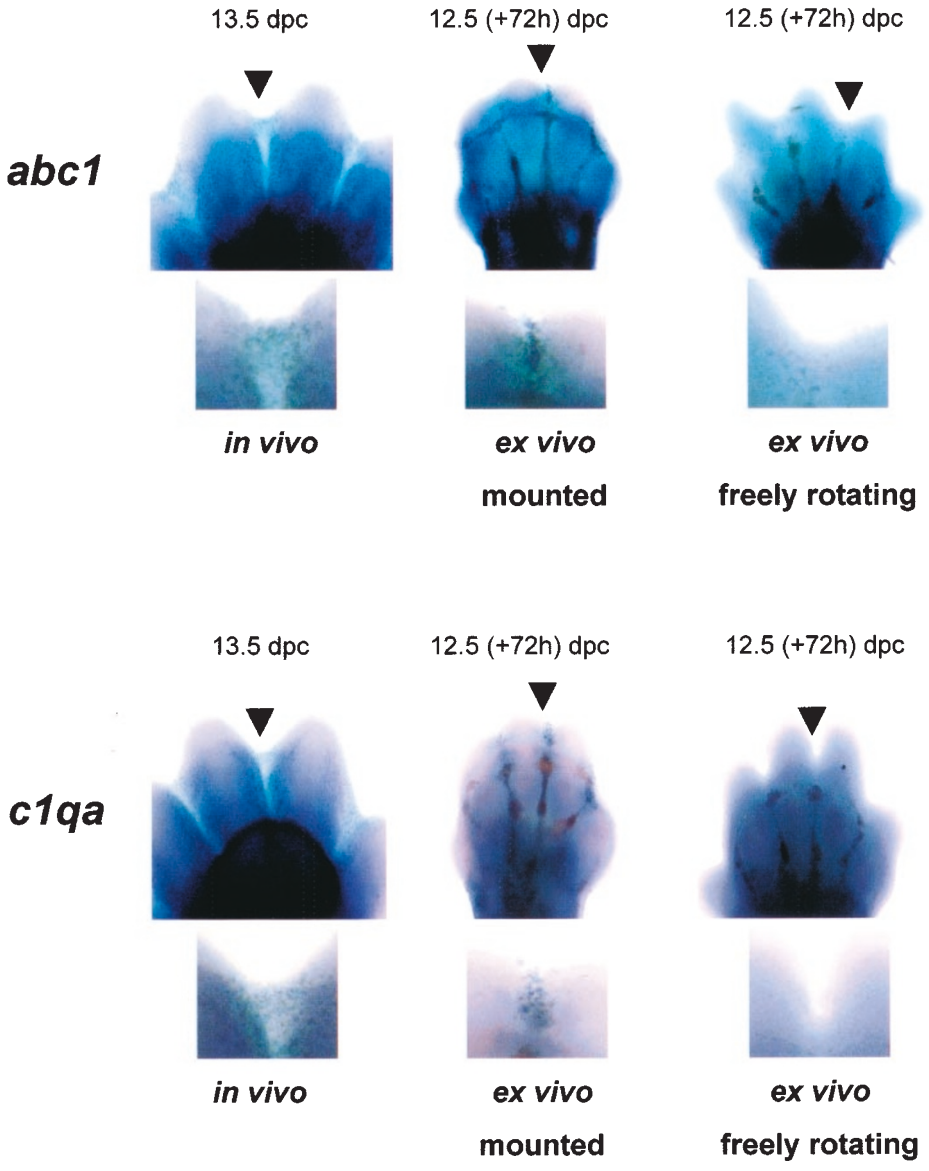

Figure 6 Presence of engulfing genes during *ex vivo* mouse limb culture as compared to its detection during development *in vivo*. Whole mount *in situ* hybridization with *abc1* (upper panel) or *c1qa* (lower panel) probes was performed on mouse forelimbs collected at 13.5 d.p.c. *in vivo* or on 12.5 d.p.c. mouse forelimbs cultured for 72 h *ex vivo* using mounted or freely rotating conditions. Arrowheads point at positive staining in interdigital tissues

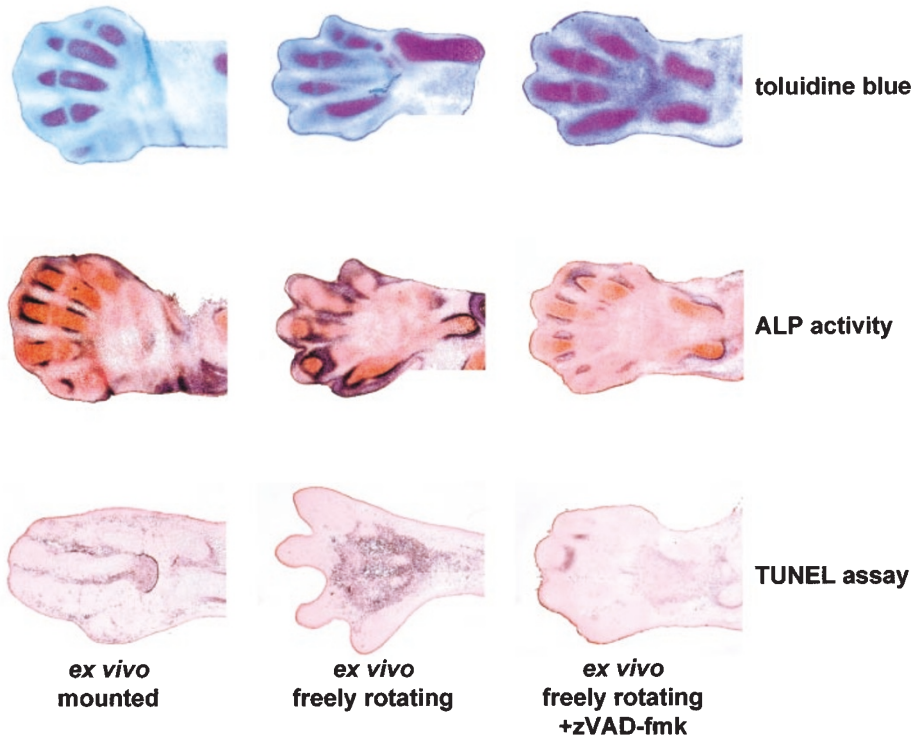

Figure 7 Caspase inhibition blocks interdigital web regression without affecting skeletal development in *ex vivo* mouse limb cultures. 12.5 d.p.c. mouse forelimbs were cultured for 72 h *ex vivo* using mounted or freely rotating conditions in the absence or presence of 20 μM zVAD-fmk and cryosectioned. Cartilage maturation and formation of joint interzones was visualized by toluidine blue staining. Onset of osteogenesis was revealed by histochemical ALP activity. Apoptotis was detected using TUNEL assay

diluted in DMSO to a 50 mM stock solution and applied as a 20 μM final concentration.

Histology, histochemical ALP activity and TUNEL assay

Mouse embryonic limbs were processed for cryostat sectioning by snap freezing in Optimal Cutting Temperature compound (Tissue-Tek®; Sakura Finetek Europe, Zoeterwoude, The Netherlands) and stored at -70°C until use. Frozen tissue sections were cut at 12 μm and fixed in 4% paraformaldehyde. Metachromatic staining of sulfated proteoglycans in the extracellular cartilage matrix was performed by toluidine blue (Fluka, Bornem, Belgium). Endogenous ALP activity, a marker for the onset of osteogenesis,[29] was detected using NBT/BCIP (Roche Molecular Biochemicals, Brussels, Belgium) as a substrate. Cells undergoing apoptosis were visualized using a TUNEL assay[31] (ALP-based *In Situ* Cell Death detection kit; Roche Molecular Biochemicals) according to the manufacturer's instructions in the presence of 2 mM levamisole (Sigma, Bornem, Belgium) to inhibit endogenous ALP activity. Except for toluidine blue stained sections, tissues were counterstained with neutral red (Klinipath, Turnhout, Belgium).

Whole mount *in situ* hybridization

Mouse embryonic limbs were fixed in MEMFA (0.1 M MOPS pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde), transferred to 90% methanol and stored at -20°C until use. Digoxigenin-11-UTP-labeled RNA probes were prepared using a DIG RNA labeling kit (Roche Molecular Biochemicals). Linearized plasmids containing cDNA of murine *col2a1*[30] (a 405 bp *Eco*RI – *Hind*III fragment of the 3′UTR; gift from Dr. Suneel Apte, Cleveland Clinic Foundation, Cleveland, OH, USA), murine *gdf5*[14] (a 1150 bp *Hind*III – *Apa*I fragment of the proregion), murine *abc1*[32] (a 1450 bp *Xba*I – *Eco*RI fragment; gift from Dr. Giovanna Chimini, Inserm CNRS, Marseille, France) and murine *c1qa*[34] (a 540 bp *Dra*I – *Bst*YI fragment; gift from Dr. Marina Botto, Hammersmith Hospital, London, UK) were applied as templates in these reactions. Whole mount *in situ* hybridization was performed using a standard protocol.[57] Probes were hybridized at 60°C and posthybridization washes were carried out to a final stringency of 0.2 × SSC at 65°C. Visualization of gene expression was obtained using an ALP-coupled anti-DIG antibody and BM Purple (Roche Molecular Biochemicals) as a substrate in the presence of 2 mM levamisole (Sigma). Prior to photography, residual background staining was cleared by rinsing the limbs in a series of 0.5% KOH in glycerol (0, 25, 50 and 75%) for 15 min each.

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