Developmental Biology ■ (■■■■) ■■■–■■■

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Evidence that the limb bud ectoderm is required for survival of the underlying mesoderm

Marian Fernandez-Teran [a], Maria A. Ros [a,b,**], Francesca V. Mariani [c,*]

[a] Departamento de Anatomía y Biología Celular, Facultad de Medicina, Universidad de Cantabria, 39011 Santander, Spain
[b] Instituto de Biomedicina y Biotecnología de Cantabria, CSIC–SODERCAN–Universidad de Cantabria, 39011 Santander, Spain
[c] Department of Cell and Neurobiology, Broad CIRM Center for Regenerative Medicine & Stem Cell Research, Keck School of Medicine, University of Southern California, Los Angeles, CA 90033, United States

ARTICLE INFO

Article history:
Received 5 May 2013
Received in revised form
21 June 2013
Accepted 25 June 2013

Keywords:
Limb development
Pattern formation
AER
Dorsal ectoderm
Cell death

ABSTRACT

The limb forms from a bud of mesoderm encased in a hull of ectoderm that grows out from the flank of the embryo. Coordinated signaling between the limb mesoderm and ectoderm is critical for normal limb outgrowth and patterning. The apical ectodermal ridge (AER), found at the distal tip, is a rich source of signaling molecules and has been proposed to specify distal structures and maintain the survival of cells in the underlying distal mesoderm. The dorsal and ventral non-AER ectoderm is also a source of signaling molecules and is important for dorsal–ventral patterning of the limb bud. Here we determine if this ectoderm provides cell survival signals by surgically removing the dorsal or ventral ectoderm during early chicken limb bud development and assaying for programmed cell death. We find that, similar to the AER, removal of the dorsal or ventral non-AER ectoderm results in massive cell death in the underlying mesoderm. In addition, although a re-epithelialization occurs, we find perturbations in the timing of *Shh* expression and, for the case of the dorsal ectoderm removal, defects in soft tissue and skeletal development along the proximal–distal axis. Furthermore, ectoderm substitution experiments show that the survival signal produced by the dorsal limb ectoderm is specific. Thus, our results argue that the non-AER ectoderm, like the AER, provides a specific survival signal to the underlying mesoderm that is necessary for normal limb development and conclusions drawn from experiments in which the non-AER ectoderm is removed, need to take into consideration this observation.

Introduction

Vertebrate limb development is considered an excellent model for developmental biology studies. The first morphological evidence of limb formation is the outgrowth of a bud from the lateral flank of the embryo. This bud consists of an accumulation of mesodermally-derived cells encased in an ectoderm hull. Critical cell–cell signaling interactions occur between these mesodermal and ectodermal components to direct outgrowth and patterning (Benazet and Zeller, 2009). In amniotes, the distal ectoderm of the limb bud adopts a thickened morphology at the dorso-ventral boundary called the apical ectodermal ridge (AER). This specialized region of ectoderm expresses a number of important signaling molecules that control limb patterning but also provides survival signals for the underlying distal mesoderm (as reviewed in (Fernandez-Teran and Ros, 2008)). Experiments carried out by John Saunders in the late 1940s showed that removal of the AER in chicken embryos causes limb truncations (Saunders, 1948). Early removal of the AER (stage 17–18HH) resulted in the most severe truncations at a proximal level, while late removal of the AER (stage 25 and later) resulted in progressively less severe distal truncations. The outcomes of this experiment were used by Wolpert and colleagues to propose the Progress Zone Model of limb patterning (Summerbell et al., 1973). Later, this model was questioned as it was shown that the AER is critical first for survival, and later for the proliferation of the subjacent mesoderm which provided an alternative explanation for the limb truncation phenotypes if proximal–distal specification occurs early (Dudley et al., 2002; Rowe et al., 1982). Thus, the AER clearly has an important role in limb development by promoting proliferation and survival of the underlying limb mesoderm.

Several members of the fibroblast growth factor (FGF) family are specifically expressed in the AER (Martin, 1998). Based on this observation, it was shown that the truncation phenotype after AER removal can be rescued by the exogenous administration of FGFs (Fallon et al., 1994; Niswander et al., 1994) suggesting that FGF are

* Corresponding author.
** Corresponding author at: Universidad de Cantabria, Departamento de Anatomía y Biología Celular, Facultad de Medicina, 1425 San Pablo St., BCC-407, MC9080, Santander 39011, United States.
E-mail addresses: rosm@unican.es (M.A. Ros), fmariani@usc.edu (F.V. Mariani).

0012-1606/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ydbio.2013.06.032

the factors from the AER that control cell survival. Four members of the *Fgf* gene family, *Fgf8*, *Fgf4*, *Fgf9* and *Fgf17*, are expressed in the AER in both mouse and chicken embryos. To study AER–FGF function, these genes have been inactivated in the mouse singly and in combination and analyzed for their contribution to limb bud development and skeletal patterning (Mariani et al., 2008; Mariani and Martin, 2003). The individual inactivation of *Fgf4*, *Fgf9* or *Fgf17* has no consequence on overall limb patterning (Colvin et al., 2001; Moon et al., 2000; Sun et al., 2000; Xu et al., 2000). However, the inactivation of *Fgf8* or, *Fgf8* in combination with any of the other AER–FGFs, results in alterations in limb pattern that can range in severity with the most extreme being a complete loss of the limb (Boulet et al., 2004; Lewandoski et al., 2000; Mariani et al., 2008; Moon and Capecchi, 2000; Sun et al., 2002). Analysis of limb bud development in different AER–FGF combination knock-out lines showed changes in limb bud size, cell survival, gene expression, and skeletal pattern that correlated with the strength of the FGF signal (Mariani et al., 2008; Mariani and Martin, 2003). Thus, in addition to providing survival and proliferative signals, the AER controls limb skeletal patterning and the AER-FGFs are critical mediators of this activity.

Besides the signaling interactions between the AER and mesoderm, the non-AER ectoderm has a role in limb patterning. In particular, the ectoderm on the dorsal and ventral sides of the limb bud is known to influence dorsal and ventral patterning. Expression of the *En1* transcription factor in the ventral ectoderm restricts *Wnt7a* to the dorsal ectoderm (Cygan et al., 1997; Loomis et al., 1998). *Wnt7a* from the dorsal ectoderm induces the expression of the homeobox gene *Lmx1b*, a homeobox-containing transcription factor responsible for establishing dorsal identity in the subjacent mesoderm (Loomis et al., 1998; Riddle et al., 1995). *Wnt7a* also influences anterior/posterior patterning by maintaining normal levels of *Shh* expression and loss of *Wnt7a* in the mouse results in dorsal to ventral transformations and in a variable loss or malformation of posterior structures, mostly digit 5 and the ulna (Parr and McMahon, 1995; Yang and Niswander, 1995). A number of studies also show that the limb ectoderm is a negative regulator of chondrogenic differentiation a function that appears to be mediated by canonical Wnt signaling (Hartmann, 2006; ten Berge et al., 2008).

In analyzing the patterns of programmed cell death in AER–FGF mutants we noticed that not only was there cell death in the proximal mesoderm of the limb bud in the AER–FGF mutants but also cell death in the proximal dorsal ectoderm (Boulet et al., 2004; Mariani et al., 2008; Sun et al., 2002) and wondered if there might be a relationship between the death of the ectoderm and the survival of the underlying mesoderm. We therefore decided to determine if, like the AER, the non-AER ectoderm also has a survival function in addition to a role in patterning, and if so, if removal of the ectoderm would impact limb bud morphological development, gene expression, and skeletal patterning.

Materials and methods

Embryos

Fertilized chicken eggs were obtained from local sources. Eggs were incubated, opened, and embryos staged following standard protocols (Hamburger and Hamilton, 1992; Ros et al., 2000). GFP-transgenic chicken embryos (McGrew et al., 2004) were locally produced at the Servicio de Estabulación y Experimentación Animal of the University of Cantabria. Mouse embryos deficient for *Fgf8* specifically in the AER were generated employing an *Msx2-Cre* transgene as described previously (Lewandoski et al., 2000; Mariani et al., 2008; Sun et al., 2002).

Removal of limb ectoderm

Right wing buds of stage 19–21HH embryos were exposed and the dorsal or ventral ectoderm was surgically removed with the aid of Nile blue sulfate (NBS) application (about one microliter of 0.15% NBS in distilled water applied over the limb surface). NBS staining not only allowed easy visualization of the ectoderm but also loosened up the ectoderm all the way to the lateral border of the somites. After a few seconds, the NBS was washed away with 1 × PBS to avoid toxicity. Then, using a fine sharpened tungsten needle, a superficial cut was made all along the junction between the dorsal or ventral ectoderm and the AER. This microsurgical technique was not used in previous studies (Yang and Niswander, 1995) and was introduced here to prevent undesired damage or detachment of the AER when peeling off the ectoderm. To compare with previous reports, the removal of the dorsal ectoderm was done without the previous cut. Removal of just the AER was done with the aid of a fine tungsten needle.

In situ hybridization, histology and skeletal preparations

Digoxigenin-labeled antisense riboprobes were prepared, and whole mount in situ hybridization performed according to standard procedures (Nieto et al., 1996). The probes used were *Wnt7a* and *Lmx1b* (Riddle et al., 1995), *Shh* (Roelink et al., 1994) and *Wnt6* (ARK genomics, ChEST972J11).

For histology, samples were routinely embedded in paraffin, sectioned and stained with Hematoxylin–Eosin. Some samples were embedded in araldite for semi-thin (1 μm thick) sections and stained with Toluidine blue according to standard protocols.

Scanning electron microscopy

Experimental and control wing buds were fixed in 2.5% Glutaraldehyde in 0.1 M-cacodylate buffer (pH 7.2), dehydrated in acetone, dried by the critical point method and then coated with gold and observed with an Inspect S microscope (FEI Company).

Cell death analysis

Cell death was detected by in situ detection of DNA fragmentation using terminal deoxynucleotidyl transferase (TdT) mediated deoxyuridine-triphosphate (dUTP) nick end-labeling (TUNEL) with the In Situ Cell Death Detection Kit, Fluorescein (Roche cat #S7110) on embryo sections or by staining whole embryos with LysoTracker Red-DND99 (Life Technologies, cat #L-7528) as previously described (Fogel et al., 2012) and subsequently sectioning them at 25 μm on a Leica vibrating microtome.

Recombinant limb experiments

Recombinant limbs were prepared by assembling operated wing buds inside limb ectodermal jackets (Ros et al., 2000). To obtain ectodermal jackets, forelimb or hindlimb buds were removed from stage 21–22HH chicken embryos and incubated at 4 °C for 60 min in 0.5% trypsin. Trypsin was inactivated by incubation in 10% chicken serum for 5 min on ice before gently separating the ectoderm from the mesoderm. In some cases, the recombinant limbs were generated by wrapping the operated limb buds with back ectoderm obtained from the dorsal flank region of stage 21–22 embryos as devised by Errick and Saunders (1976). The back ectoderm was obtained following the same procedure as for the ectodermal jackets. The recombinant limbs were grafted to the paraxial mesoderm of stage HH20–22 host embryos.

Please cite this article as: Fernandez-Teran, M., et al., Evidence that the limb bud ectoderm is required for survival of the underlying mesoderm. Dev. Biol. (2013), http://dx.doi.org/10.1016/j.ydbio.2013.06.032