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Review article

Embryology meets molecular biology: Deciphering the apical ectodermal ridge

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ABSTRACT

More than sixty years ago, while studying feather tracks on the shoulder of the chick embryo, Dr. John Saunders used Nile Blue dye to stain the tissue. There, he noticed a darkly stained line of cells that neatly rims the tip of the growing limb bud. Rather than ignoring this observation, he followed it up by removing this tissue and found that it led to a striking truncation of the limb skeletons. This landmark experiment marks the serendipitous discovery of the apical ectodermal ridge (AER), the quintessential embryonic structure that drives the outgrowth of the limb. Dr. Saunders continued to lead the limb field for the next fifty years, not just through his own work, but also by inspiring the next generation of researchers through his infectious love of science. Together, he and those who followed ushered in the discovery of fibroblast growth factor (FGF) as the AER molecule. The seamless marriage of embryology and molecular biology that led to the decoding of the AER serves as a shining example of how discoveries are made for the rest of the developmental biology field.

1. Saunders' seminal work on the properties of the AER paved the way for the race to discover the molecular identity of the AER molecules

Dr. John Saunders' early embryological work set the foundation for the field of limb development. Limbs originate as buds from two set positions along the sides of the embryo, forming the pairs of forelimbs and hindlimbs. Early limb buds consist of undifferentiated mesenchymal cells surrounded by an epithelial jacket. Signaling between these two tissues is essential for the development of the limb along its three axes: Anterior-Posterior (A-P), Dorsal Ventral (D-V), and Proximal-Distal (P-D). Focusing on the P-D axis, it is divided into three main segments, the stylopod (humerus, femur), the zeugopod (radius/ulna, fibula/tibia), and the autopod (wrist/digits, ankle/toes). Through elegant manipulations of the chick limb buds in ovo, Dr. Saunders uncovered the key principles of limb development that guided the field for many years to come.

Originally studying the properties of feather tracts on the shoulder of birds, Dr. Saunders noticed that when he used the vital dye Nile Blue Sulfate to enhance tissue visibility in the embryo, the dye stained the apex of the limb bud ectoderm. This ectodermal thickening that rims the distal tip of the limb bud is what came to be known as the Apical Ectodermal Ridge (AER). To investigate the properties of the AER, in a meticulous set of in ovo surgical manipulations, Dr. Saunders removed the AER at various limb bud stages, and then returned the eggs to incubation until limb skeletons have developed. To his surprise and delight, he discovered that removal of AER led to loss of terminal limb skeletal elements. The earlier the AER is removed, the more the limb skeleton is truncated along the P-D axis (Saunders, 1948). This experiment led to two main conclusions: that the presence of the AER is required for continued outgrowth of the limb; and the limb segments are developed in a P-D sequence.

Others quickly built on the findings from Saunders. For example, Dr. Edgar Zwilling examined a wingless strain of bird, observing that the AER in this mutant degenerated during development, likely the reason for the absence of the limb skeleton. He also noted that when mutant mesenchyme was placed inside a wild-type ectodermal jacket, the wild-type AER quickly degenerated. This provided the first evidence that a factor from the mesenchyme was required to maintain the AER (Zwilling, 1949). These additional experiments corroborated Dr. Saunders' conclusion that the AER is essential for limb outgrowth and development.

In 1948, the finding that the epithelium plays an essential role in achieving the limb pattern was in contrast to the common belief that it is just a passive tissue to encase the mesenchyme. Over the next two decades, many experiments were performed aimed to challenge the importance of the AER (Saunders, 1998). However, those studies, some of which involved Dr. Saunders himself, all came back with the

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overriding conclusion that the AER is key for limb P-D outgrowth. For example, Saunders and Gasseling showed that when an AER was grafted to the dorsal or ventral side of the limb epithelium, the limb bud that had two AERs gave rise to two limbs, suggesting the AER is sufficient at inducing limb outgrowth (Saunders and Gasseling, 1968). This conclusion led to several new questions. Is the mesenchyme from the hindlimb able to respond to the AER from a forelimb? Does the AER determine whether a limb mesenchyme will form a forelimb or a hindlimb? Are the signals from the AER species specific? To address these, Gasseling and Saunders grafted forelimb epithelium to hindlimb mesenchyme and vice versa which demonstrated that the limb bud mesenchyme could respond to either AER for outgrowth, but it is the mesenchyme that determined whether a wing or a leg was made (Gasseling and Saundersg, 1961). Subsequent grafting experiments combining duck leg mesenchyme and chick epithelium gave rise to a foot with webbing (Zwilling, 1959; Saunders and Fallon, 1967). Similarly, grafting turtle ectoderm to chick mesoderm resulted in efficient outgrowth of limbs with characteristics of the chick. These results demonstrate that the ability of the AER to drive outgrowth is conserved across species, and the type of limb structures that form is dictated by the mesenchyme (Saunders, 1998).

Two experiments by Errick and Saunders sought to address the question of whether the structural configuration of the AER was important for its function. First, when the epithelial hull of a limb bud was removed and turned inside out and regrafted, P-D development occurred normally (Errick and Saunders, 1974). Also, if the AER was removed and the cells dissociated, re-aggregated and placed back at the apex of the limb bud, they will form a new ridge and induce proper P-D development (Errick and Saunders, 1976). These experiments suggest that whatever signal the AER sends to the mesenchyme, it is still present and functional even if the ridge structure is reorganized.

Another important aspect of the AER uncovered by Rubin and Saunders was that the AER is permissive for outgrowth, and does not specify the P-D sequence of limb segments. This was demonstrated by grafting old AERs onto young limb bud mesenchyme, and vice versa, to show the limb buds always develop the correct P-D sequence of limb segments appropriate to the stage of the component mesenchyme (Rubin and Saundersg, 1972). Therefore, the function of the AER is the same in a young limb bud compared to an older, more developed limb bud.

Subsequent experiments analyzing cell survival after ridge removal showed a region of mesenchymal cell death 150–200 μm underneath the AER (Rowe et al., 1982; Dudley et al., 2002). This cell death was only observed when the AER was removed early during limb bud development. When the AER is removed after stage 25, P-D limb truncation still occurred even though no cell death is observed, demonstrating that while the AER is required for P-D outgrowth at all limb bud stages, it is only required for cell survival early in limb bud development. Altogether, these pioneering embryological studies established a rich knowledge base, and set the stage for the hunt for the molecular identity of the AER function.

2. Discovery of the AER molecule(s): embryology meets molecular biology

Starting in the late 1970s, the wave of advancements in molecular biology led to the development of many new tools to uncover the molecular identities of the activities defined in classical embryological and physiological experiments. Among these tools, in situ hybridization became an indispensable technology to determine the specific mRNA expression patterns of genes in the embryo. For AER activity, given its non-cell-autonomous effect on the subjacent mesenchyme, attention had been on secreted factors. Several sets of secreted factor genes show localized expression in the AER. These include Fibroblast Growth Factor genes (Fgfs), Bone Morphogenetic Proteins genes (Bmps), and Wingless homolog genes (Wnts) (Niswander and Martin, 1992; Suzuki et al., 1992). For example, *Fgf2* and *Fgf4* were the first Fgfs shown to be expressed in the AER of developing chick or mouse limb buds (Savage et al., 1993; Niswander and Martin, 1992).

The first line of evidence for a candidate AER molecule came when studies showed that FGF could promote mesenchymal cell proliferation, inhibit cell death and differentiation in either dissociated limb mesenchyme or limb bud organ culture after AER removal (MacCabe et al., 1991; Munaim et al., 1991). The definitive test of FGF as the AER activity came independently from two groups, each combined molecular biology with Saunders' style embryology (Niswander et al., 1993; Fallon et al., 1994). It was found that when PBS or FGF4 soaked beads were implanted onto AER denuded limb buds, the FGF4 bead, but not PBS bead, can nicely replace AER function. Cell death was prevented in the underlying mesenchyme. As a result, all three segments of the limb skeleton were rescued by FGF after AER removal. These studies offered definitive demonstration that FGF signaling is sufficient to serve as the AER activity to promote P-D outgrowth. Defining the molecular players in limb outgrowth was a major leap forward in the field of limb development.

3. Genetic demonstration of AER function: leading the wave for conditional gene knockouts

While chick is an excellent system to perform gain-of-function or activity replacement studies to address sufficiency, it is not an easy setting to test necessity in a gene-specific manner. For the latter, the field turned to use the gene knockout approach in mice. Of the Fgf genes expressed in the AER, the most prominent ones with a restricted pattern in the AER are *Fgf4* and *Fgf8*. However, the test for their necessity in driving limb bud outgrowth was complicated by the findings that each of these Fgf genes has important roles in early embryogenesis prior to limb development. Global knockout of either *Fgf4* or *Fgf8* led to lethality at peri-implantation or gastrulation stages, respectively (Feldman et al., 1995; Sun et al., 1999). Thus, to study their requirement in limb development, a conditional gene knockout approach is needed.

In a case of serendipity, Gail Martin's laboratory, which is one of the labs that first demonstrated the sufficiency of FGF function in the AER in chick, was also at the forefront of developing the *Cre-loxP* mediated conditional knockout approach in mice. In the Martin lab, at the same time when Dr. Lee Niswander operated on the AER in chick embryos, one bench over, Dr. Mark Lewandoski was testing DNA constructs for generating *Cre-loxP* mediated deletions of genomic DNA (Niswander et al., 1993; Lewandoski et al., 1997). This naturally led to the use of Fgf genes as a test case for tissue-specific inactivation. Floxed (flanked by *loxP*) alleles of *Fgf4* and *Fgf8* were generated, each with small 34 basepair *loxP* sequences inserted into introns or the 3′ untranslated regions, flanking critical exons (Meyers et al., 1998; Sun et al., 2000). At the same time, an 139 base-pair piece of *Msx2* promoter was used to drive limb ectoderm-specific expression of Cre (Liu et al., 1994; Sun et al., 2000). The combination of *Msx2-cre* and Fgf floxed alleles led to efficient gene inactivation in the AER.

Based on gene expression pattern, and FGF bead implantation experiments in chick, the most likely FGF to execute AER function was FGF4. Thus, it came as a major surprise that a clear inactivation of *Fgf4* in the limb bud led to no limb skeletal defects (Sun et al., 2000). Furthermore, while inactivation of *Fgf8* in the limb bud led to skeletal reductions, the defects are rather mild compared to that seen after AER removal (Lewandoski et al., 2000; Moon and Capecchi, 2000). In addition to *Fgf4* and *Fgf8*, it was found that *Fgf9* and *Fgf17* are also expressed in the AER in mice (Sun et al., 2000). However, individual inactivation of neither *Fgf9* nor *Fgf17* led to limb defects (Mariani et al., 2008). These results led to speculations that either FGFs are not the major AER factors, or that there is substantial redundancy of function among Fgfs expressed in the AER.