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Identification of genes expressed in the mouse limb using a novel ZPA microarray approach

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Abstract

One well-studied signaling center in the developing vertebrate limb, the zone of polarizing activity (ZPA), produces the morphogen sonic hedgehog that is necessary for normal growth and pattern formation. To identify additional factors expressed in the ZPA of the mouse limb bud, the *Shhgfpcre* allele was used to purify ZPA cells using fluorescence-activated cell sorting. Microarray technology was then used to identify genes whose expression was elevated in the ZPA compared to the rest of the limb. *In situ* hybridization confirmed the expression of two known transcription factors, *Hlxb9* and *Tcfap2b*, an uncharacterized EST, and a transmembrane protein of unknown function in domains overlapping the ZPA. The expression of two other genes was confirmed by rtPCR. The methods described in this report will be applicable for identifying genes enriched in *Shh*-expressing cells throughout development.

Keywords: ZPA; Shh; Tmem16a; Limb; Mouse; Microarray; Affymetrix; FACS; Tcfap2b; Hlxb9; Ppp1cb; Ywhaz

1. Results and discussion

For decades, scientists have investigated the complex mechanisms that regulate development of the vertebrate limb. In a classical experiment, Saunders and Gasseling grafted a population of mesenchymal cells from the posterior of one chick limb bud to the anterior of a recipient chick limb bud (Saunders and Gasseling, 1968). This manipulation resulted in supernumerary digits that were patterned so that those closest to the anterior site of the graft assumed a more posterior identity. In this manner, the zone of polarizing activity, or ZPA, was identified as a signaling center that is necessary for the normal anteroposterior patterning of the vertebrate limb.

The ZPA was hypothesized to function by secreting a morphogen that established a gradient along the anteroposterior axis with different concentrations of the unidentified morphogen specifying digit identities (Wolpert, 1969). Sonic hedgehog, a homolog of *Drosophila hedgehog*, was identified as the ZPA morphogen (Riddle et al., 1993). *Shh* expression in the limb is confined to the domain shown to possess polarizing activity in ZPA grafting experiments. Furthermore, when fibroblasts expressing *Shh* were transplanted to the anterior of the limb, mirror-image duplications reminiscent of those seen in ZPA grafts were observed. Genetic knockout in mice demonstrated that *Shh* functions not only to pattern the anteroposterior axis of the limb bud, but also plays a role in regulating the outgrowth of the limb (Chiang et al., 1996).

A second signaling center, the apical ectodermal ridge (AER), is also required for the outgrowth of the limb (Saunders JW, 1948). Fibroblast growth factors (FGFs) expressed in the AER are necessary for the maintenance of *Shh* expression in the ZPA (Niswander et al., 1994). Conversely, SHH in the ZPA is capable of regulating *Fgf4* expression and functions in a positive feedback loop to promote outgrowth of the limb (Laufer et al., 1994; Niswander et al., 1994). The bone morphogenetic protein (BMP) antagonist *gremlin* is induced in the limb bud

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mesenchyme in response to low levels of BMP2 (Nissim et al., 2006) and prevents the *Bmp*-mediated regression of the AER (Ganan et al., 1998). It has recently been shown that descendants of *Shh*-expressing cells do not express *gremlin* (Scherz et al., 2004). As the limb bud grows, a wedge of *gremlin*-negative cells forms between the *Shh*-expressing cells of the ZPA and the *Fgf*-expressing cells of the AER. This results in the eventual breakdown of the *Shh–Fgf* feedback loop and the termination of limb outgrowth.

By irreversibly marking the descendants of *Shh*-expressing cells using the *Shhgfpcre* allele (Harfe et al., 2004) and a *lacZ* reporter allele (Soriano, 1999), we have previously shown that the most posterior digits arise from cells that expressed *Shh* in the ZPA (Harfe et al., 2004). The identities of digit 4 and digit 5, which are completely derived from cells that have expressed *Shh*, are specified by the duration of their exposure to SHH. Cells giving rise to digit 5 are exposed to high concentrations of SHH for a longer period of time than those giving rise to digit 4. In contrast, specification of digit 2 is determined only by the concentration of SHH that has diffused across the limb field. Digit 3 is composed of a mixture of cells, some that have actively expressed *Shh* and some that have only responded to SHH protein (Harfe et al., 2004). Consistent with the data from *Shh* null mice (Chiang et al., 1996), digit 1 specification is *Shh*-independent.

To date, *Shh* is the only gene known to be specifically expressed in the ZPA of the vertebrate limb. In this report we describe experiments designed to identify additional genes expressed in the ZPA that function either in conjunction with SHH or independently to pattern the vertebrate limb. These factors might be involved in the secretion or reception of SHH, serve as a cellular memory of previous exposure to SHH, regulate proliferation and/or differentiation in the limb or function in some other unforeseen pathways.

To identify genes expressed in the mouse ZPA, we used the *Shhgfpcre* allele (Harfe et al., 2004) in combination with fluorescence-activated cell sorting (FACS) to purify two populations of cells from the developing mouse limb: one from the ZPA and one from the rest of the limb (Fig. 1). From these populations, labeled cRNA was synthesized and hybridized to Affymetrix GeneChips to identify genes differentially expressed between the ZPA and the rest of the limb. Analysis of the microarray data led to the identification of four genes whose expression overlaps the ZPA.

1.1. An in vivo screen to identify genes expressed in the mouse ZPA

To purify ZPA cells from the limb, limb buds were dissected from *Shhgfpcre*-heterozygous embryos (Fig. 1). Mice heterozygous for this allele appear wild type and express green fluorescent protein (GFP) as well as the bacterial recombinase Cre in all cells that express *Shh*. After dissociating the limbs into single cells, GFP-positive cells (the ZPA) and GFP-negative cells (the rest of the limb) were

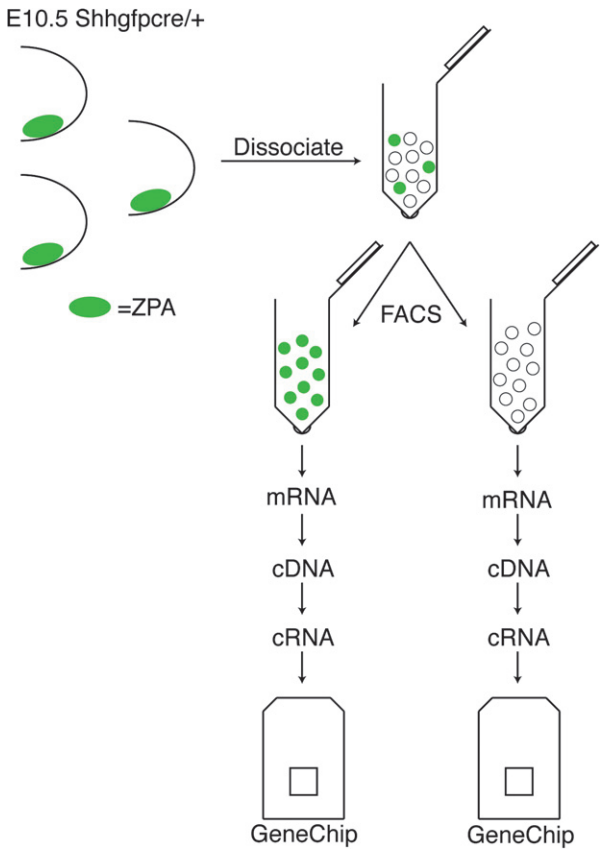

Fig. 1. Analysis of gene expression in *Shh*-expressing cells of the ZPA and the rest of the E10.5 limb bud. Limbs were dissected from E10.5 *Shhgfpcre* heterozygous embryos and dissociated into single cells (see Section 2). GFP-positive (ZPA) and GFP-negative (rest of the limb) cell populations were purified by FACS. From these populations, labeled cRNA was synthesized and hybridized to Affymetrix GeneChips to compare whole-genome expression between cells of the ZPA and the cells of the rest of the limb. A total of eight GeneChips were used: four with purified ZPA cells and four with cells from the rest of the E10.5 limb bud.

purified using fluorescence-activated cell sorting (FACS). Biotin-labeled cRNA was synthesized from each population of cells and hybridized to Affymetrix GeneChips. Analysis of the microarray data identified a number of genes that are differentially expressed in the ZPA and the rest of the limb (Table 1, Supplementary Table 1, and data not shown). The raw data is available from the Gene Expression Omnibus of the National Center for Biotechnology Information (accession number GSE7598).

Since our screen was designed to identify genes whose expression was enriched in the ZPA, we expected that *Shh* would be detected at higher levels in the GFP-positive cells. Indeed, this was the case; our array data indicated that *Shh* transcripts were more than 20 times as abundant in the ZPA cells than in the rest of the limb (Supplementary Table 1). Other genes previously described as being present in the ZPA were also identified by our screen. For example, *Gli1*, *Bmp2* and *Hand2* were all detected at higher levels in the ZPA than in the rest of the limb. Our microarray data analysis of genes previously described as being expressed in