

Chapter 9

Electroporation into the Limb: Beyond Misexpression

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1 Introduction

Limb development has been studied for over 100 years by several generations of developmental biologists. The developing limb is one of the best models with which to study pattern formation in vertebrates. We have used chick limb development to answer a simple but basic question, namely, why heterogeneous tissues are formed at correct positions and times from a homogeneous population of cells (Pearse & Tabin, 1998).

Limb development starts as two pairs of tissue bulges in the lateral plate mesoderm (LPM). These are called the forelimb and hindlimb fields (Fig. 9.1). After limb initiation, one can clearly identify three-dimensional axes in the limb buds: the proximal-distal (PD; from shoulder to fingers), dorso-ventral (DV; from back to palm), and antero-posterior (AP; from thumb to little fingers) axes. Morphological changes and differences along these three axes are determined by pattern formation during limb bud stages. Following establishment of these axes, one can visually recognize condensation of cartilages. Muscles, tendons, and neurons migrate and differentiate after cartilage formation. Because the stages and events are easily recognized morphologically and in detail, it is therefore the limb bud is an excellent model with which to study the molecular mechanisms of embryonic patterning and tissue differentiation in vertebrates.

1.1 *History of Basic Experimental Strategy*

One of the most fundamental discoveries in the study of limb development was based on the pioneering use of tissue transplantation within limb buds. In 1968, John Saunders Jr. discovered cells in ZPA (zone of polarizing activity) located

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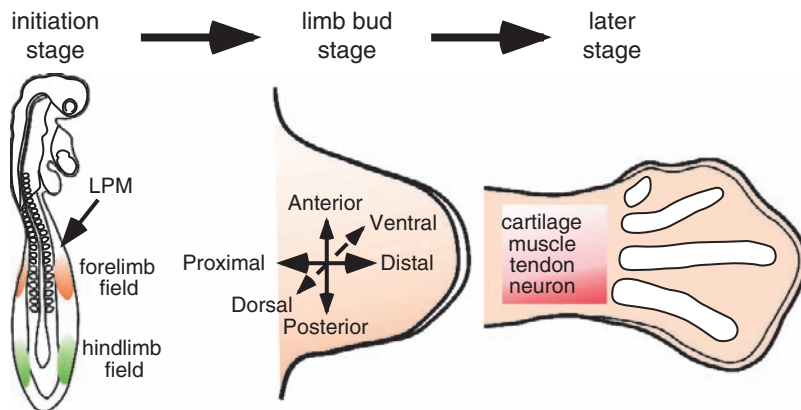


Fig. 9.1 Chick Limb bud development. Forelimb and hindlimb start to develop at the lateral plate mesoderm (LPM). These fields are called the forelimb field and the hindlimb field. After limb initiation, the three axes of the limb bud are specified: antero-posterior axis, proximo-distal axis, and dorso-ventral axis. At later stages, cartilage starts to condense. Subsequently, muscle, tendon, and neuron migrate into the limb and differentiate (See Color Plates)

at the posterior edge of the limb bud (Saunders & Gasseling, 1968). When he transplanted ZPA cells to the anterior side of the limb bud, a mirror-image duplication of digits was induced in the resulting limb. In addition, the digits induced at the anterior side had posterior digit identities. This result showed that there must be a gradient of diffusible molecule(s) which determine positional information along the AP axis of the limb bud, with a higher concentration in the posterior region and a lower one in the anterior. In 1993, it was reported that the *Shh* gene is expressed in the ZPA region (Riddle et al., 1993) (Fig. 9.2). SHH is a secreted molecule, and implantation of beads soaked in SHH at the anterior side of the limb bud induced the same mirror-image duplication as that observed for the transplantation of the ZPA cells (López-Martínez et al., 1995). Thus, SHH was proposed to be a morphogen expressed at the posterior side of the limb bud and which specifies positional values along the AP axis.

In addition to *Shh*, they have been identified, many other secreted molecules with the ability to influence limb pattern were identified. *Fgf8/Fgf4*, expressed in the AER (apical ectodermal edge), and *Fgf10*, expressed in the mesoderm, were both found to be necessary for the outgrowth of the limb bud along the PD axis (Ohuchi et al., 1997; Sun et al., 2002). *Wnt7a* was found to be expressed only in the dorsal ectoderm, and *Wnt7a* knockout mouse showed ventralization of the limb. Thus, it was inferred that *Wnt7a* specifies the dorsal limb bud identity (Parr & McMahon, 1995). As for *Shh*, these and other signaling molecules can easily be studied by implantation of protein-soaked beads. A second technique easily used for the study of patterning molecules in the limb bud is the expression of transgenes, described below.

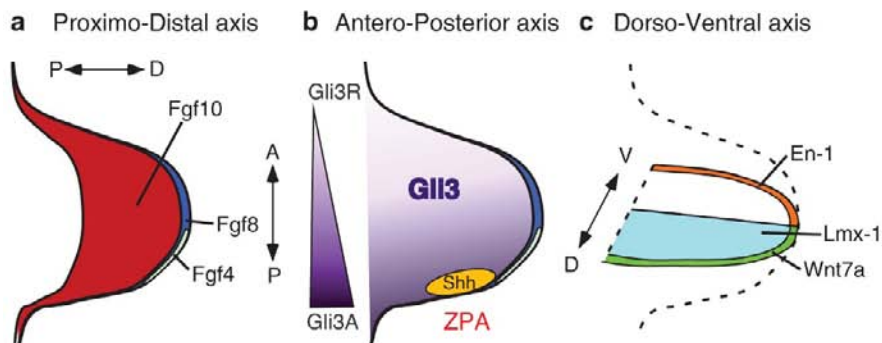


Fig. 9.2 Three axes in the limb bud. *Fgf8* and *Fgf4* are expressed at the apical ectodermal ridge (AER). In contrast, *Fgf10* is expressed only in the mesoderm. They are each necessary for the other to maintain its expression during limb bud outgrowth along the proximo-distal axis. *Shh* is expressed in the posterior limb bud and establishes antero-posterior limb bud identity through the transcription factor *Gli3*. SHH signaling inhibits degradation of *Gli3*, and leads to the formation of full length *Gli3*, called *Gli3A*, in the posterior. On the other hand, *Gli3* is degraded in the anterior, producing a short form of *Gli3*, called *Gli3R*. Thus, a *Gli3R*–*Gli3A* gradient exists along the antero-posterior axis on the basis of SHH activity. *Wnt7a* is expressed in the dorsal ectoderm, and *Lmx-1* is expressed only in the dorsal mesoderm. In contrast, *Engrailed-1* (*En-1*) is expressed in the ventral ectoderm, where it inhibits *Wnt7a* and *Lmx-1* expression. This combination establishes dorso-ventral polarity (See Color Plates)

2 Procedure

Here we detail of protocols to express transgenes into limb by electroporation.

2.1 Preparation of Egg and DNA Solution

Incubate eggs in a humidified incubator (IC800, Yamato) at 39°C until St. 13/14, kept down for easy access. An 18G1/2 needle (NN-1838R, Terumo) or forceps (Dumont No. 5 forceps, 11 cm; No. 500341, World precision instruments) should be used to create a window at the broader edge of the eggshell. ~5 ml of albumin should be gently removed from this hole with a 25 ml syringe (SS-20ESZ, Terumo). Using this procedure, one can procure enough space inside the egg for electroporation.

The pCAGGS (Niwa et al., 1991) or RCASBP (Logan & Tabin, 1998) vector is next diluted with distilled water to a final concentration of 3 to 5 µg/ml and mixed with pCAGGS-EGFP expression vector (0.8 to 1 µg/ml) for electroporation. This concentration of the pCAGGS-EGFP vector is enough to visualize the entire electroporated area (Momose et al., 1999). A cocktail of expression plasmids can then be placed on the cap of a PCR tube (0.2 ml), and mixed with a solution

of 1% Fast Green (diluted in PBS; No. 061–00031, Wako) before electroporation. This electroporation cocktail is then vacuum-extracted by a glass capillary attached to an aspirator tube.

2.2 Injection of DNA Solution into Limb Fields

After opening an eggshell, one can observe the chick embryo under the vitelline membrane. At this moment, a small amount of a Rotring-PBS solution (PBS with 1:40 Rotring; Art-R 591017, Sanford) (approximately 100 to 200 μ l) is injected from outside of the sinus terminalis located near the tail bud, using a syringe equipped with a 26G1/2 needle (NN-2613S, Terumo). After bathing the embryo in 1 ml of sterilized PBS, vitelline membrane near either the forelimb or hindlimb field is gently shorn by a sharpened tungsten needle. At St. 14 (the best stage for electroporation into limb buds) 22 pairs of somites are present (Fig. 9.3a). DNA solution is injected into an embryonic space located between the somatic lateral plate mesoderm (LPM) and the splanchnic LPM. An L-shaped platinum cathode is inserted from the hole that was created for injection of the Rotring-PBS solution (Fig. 9.3b), and placed under either the forelimb or hindlimb field (Fig. 9.3c–e).

To inject DNA solution into the forelimb field, the tip of a glass capillary tube is inserted from the anterior side of the forelimb field by pricking the thin embryonic tissue. For injection into the hindlimb field, a glass capillary tip is inserted from the posterior side of the hindlimb field, careful to avoid any damage to the vitelline artery, which typically results in the death of embryo. After insertion of the needle, DNA solution can be injected. After successful injection, one can observe green pigment in the Fast Green-filling forelimb or hindlimb field.

2.3 Electroporation into Limb Fields

We developed three different methods by modifying our original protocol (Ogura, 2002), typically using a platinum anode (Fig. 9.3f).

1. When an anode is placed at the center of either the forelimb or the hindlimb field before electric pulses (8 V, 60 ms pulse-on, 50 ms pulse-off, three repetitions), one can express transgene along the entire limb bud, with strong expression along the middle part of the limb. By positioning an anode at either the anterior side or the posterior side of the limb field, transgene expression becomes restricted to the anterior or posterior portions, respectively.
2. One can expand the domain of transgene expression by moving an anode serially. In this case, the electroporator is set for three pulses (8 V, 60 ms pulse-on, 1 second pulse-off, for three repetitions). For the first pulse, an anode is placed in the anterior limb field. During a 1 s pulse-off pause, this anode is moved to the

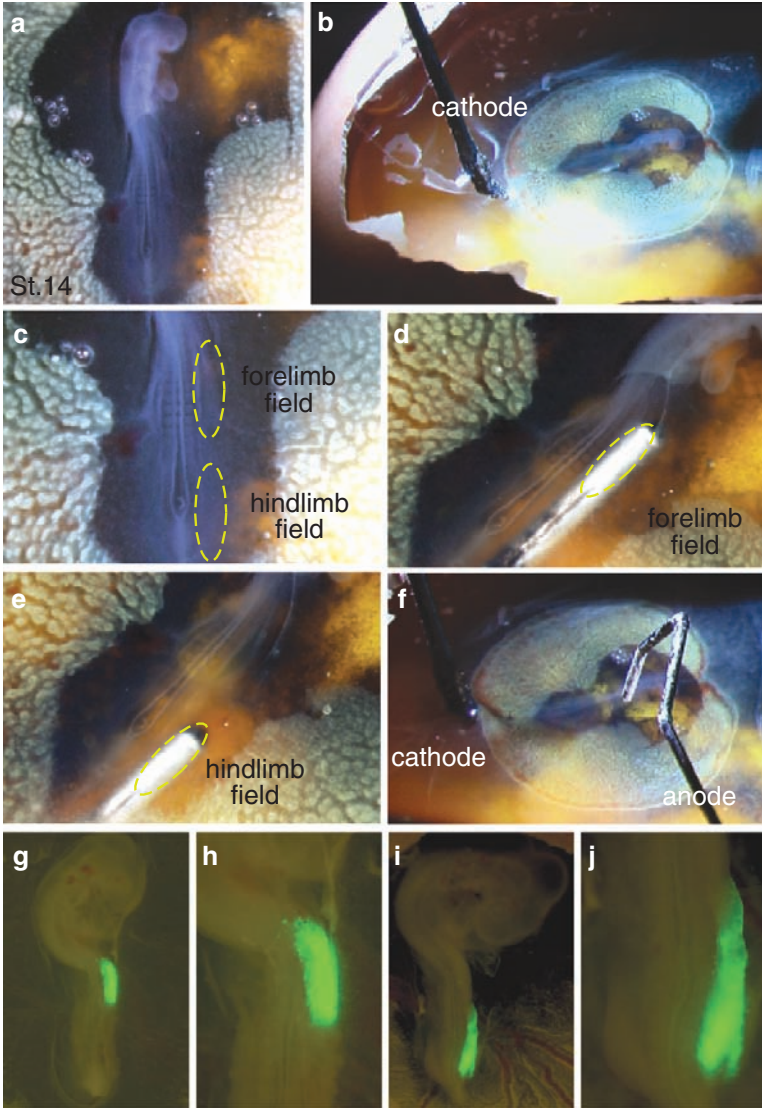


Fig. 9.3 Electroporation into the chick limb field. **(a)** After injection of Rotring-PBS solution, a St. 14 chick embryo is highlighted on the black background. **(b)** A cathode electrode is inserted under the embryo. **(c)** Forelimb and hindlimb fields are shown by a yellow circle. A cathode electrode is placed under the forelimb field **(d)** or hindlimb field **(e)**. **(f)** An anode electrode is placed in the forelimb or hindlimb field. **(g–j)** $5\ \mu\text{g}/\mu\text{l}$ of pCAGGS-EGFP was electroporated into the forelimb field **(g, h)** or hindlimb field **(i, j)**. EGFP expression is detected at 12h after electroporation. **h** and **j** show high magnification of **g** and **i**, respectively (*See Color Plates*)

central part of the limb field, and then a second pulse is applied. The anode is moved further to the posterior limb field for the last electric pulse. By applying three electric pulses serially in different parts of the limb field, one can over-express transgene strongly and uniformly in the whole limb bud.

3. An anode can be placed over the limb field, making a parallel configuration with a cathode for electric pulses (5 V, 60 ms pulse-on, 50 ms pulse-off, three repetitions). In this setting, one can misexpress transgene throughout the entire limb bud. However, lower voltage pulses must be employed, since the electric field formed between two parallel electrodes is wider and a higher voltage in this setting may damage cells.

During electroporation, two electrodes must be kept in solution, not touching the surface of the embryos or the vitelline artery in order to avoid tissue damage. When truncation or shortening of the limb buds is observed, even after electroporation with non-toxic pCAGGS-EGFP (Fig. 9.3g–j), this finding must be replicated in order to confirm the electrodes were free from the embryonic tissues, and not affecting the experimental results.

After electroporation, the cathode is gently withdrawn from the amnion, and 30 μ l of a penicillin-streptomycin solution (PBS with 1:100 penicillin-streptomycin; No. 15140–122, Invitrogen) (for 1 L 1xPBS: NaCl 5.8 g, $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ 0.36 g, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 2.76 g) is used to bathe the embryo. The eggshell window is then sealed firmly with plastic tape. Embryos should be incubated again immediately after sealing.

3 Comments

3.1 *Retrovirus and/or Electroporation*

In 1992, Morgan et al. showed that misexpression of *Hoxd11* in the chick hindlimb induced posteriorization of digit 1 to digit 2 (Morgan et al., 1992). To overexpress *Hoxd11*, they used the replication-competent retrovirus system (RCASBP). Thus, they introduced a transcription factor into a whole limb bud by retrovirus infection. This experiment made a large impact on chick developmental biology, proving that efficient and widespread misexpression of genes in the limb bud is powerful enough to induce dramatic morphological changes. By using this method, many transcription factors and diffusible proteins were identified as patterning molecules. For example, overexpression of *Lmx1* – a transcription factor normally expressed only in the dorsal mesoderm – was found to result in the dorsalization of muscle and tendon structures in the chick (Riddle et al., 1995). In contrast, misexpression of *Engrailed-1* – normally expressed in the ventral ectoderm – was found to inhibit the expression of *Wnt7a* and *Lmx1* to establish the ventral identity of the limb bud (Logan et al., 1997).

At later stages, *BMPs* (bone morphogenic proteins) 2, 4, 5, 7, and *GDFs* (growth and differentiation factors) 5 and 6 are expressed around cartilage, and implantation of beads soaked in these proteins promotes differentiation of chondrocytes (King et al., 1996). Similarly, overexpression of a dominant negative BMP receptor 1B by retrovirus infection inhibited its differentiation (Zou et al., 1997). In contrast, misexpression of the transcription factor *Pax3* induced ectopic expression of *MyoD* and subsequent differentiation of muscles at later stages (Bendall et al., 1999).

As evidenced by these results, the retrovirus system is a useful tool for the study of pattern formation and cell differentiation. Although the retrovirus system is powerful in its ability to deliver exogenous DNAs (Morgan & Fekete, 1996), there are some restrictions to its use (Table 9.1). First, the sizes of transgenes need to be less than 2.4 kb in the RCAS system, since longer transgenes inhibit efficient packaging of retrovirus, thereby resulting in a lower titer. Second, it is difficult to obtain high titer viruses when transgenes are toxic to the chick embryonic fibroblasts. Such transgenes include those that inhibit the cell cycle or induce apoptosis.

The biggest disadvantage of this system is the time lag between the injection of virus and the onset of gene expression. After injection, chick cells are infected and a viral genome is integrated into the host genome. Following viral integration, transcription of the integrated transgene initiates, and synthesis of the protein product becomes evident 20h after injection of the virus solution. Once expression of the viral genome starts, viral particles infect surrounding cells, resulting in spatial expansion of gene expression to the entire limb bud. Although infection is straightforward, expansion throughout the limb bud takes a relatively long time (2 days). Therefore, high virus titers are necessary for robust and extensive expression of transgenes at early stages, such as during initiation of limb outgrowth.

Although retroviral infection is useful, electroporation offers several advantages over the retroviral approach that make it even more powerful and effective (Ogura, 2002). First, there is almost no limit to the size of transgenes, since expression of electroporated transgenes does not require their packaging into viral particles.

Table 9.1 Comparison of electroporation and virus infection

	Electroporation	Virus infection
Size of transgene	No limit	<2.4 kb
Expression level	High	Low
Vector	No limit	RCAS, RCAN
Expression starts from	<3 h	<12 h
Expression area	Whole limb or restricted area	Whole limb
Visualization of experimental area	Yes	Difficult but possible
Damage to tissue	High	Low
Suitable experimental stage	From limb initiation stage	From limb bud stage
Suitable transgene	Transcriptional factor receptors, adaptor protein	Small protein less than 2.4 kb secreted protein

Second, we can use powerful exogenous promoters, such as the CMV or the CMV promoter-based chick beta-actin promoter, to drive expression. Electroporation also allows for a wide range of choice of vector systems. In the limb bud, the pCAGGS vector exhibits a 10 times stronger expression than the RCAS vector. The Xenopus elongation factor promoter exhibits a similar strength in the chick limb bud (Suzuki et al., unpubl. data, 2008). Third, electroporated transgenes are transcribed immediately and expressed as proteins within 3 h. When the RCAS system is used, it takes 20 h for any significant expression of transgenes. In our hands, expression of EGFP at a high level is seen within 6 h after electroporation when the pCAGGS-EGFP expression plasmid is used. This rapid expression of transgenes enables us to study events even at very early stages, such as during the initiation of limb outgrowth. Fourth, in contrast to the wide expansion of transgene expression that occurs with the RCAS system, one can misexpress transgenes in a restricted subdomain of the limb at any position, such as a small portion of the anterior side of the limb. In the case of the RCAS retrovirus system, it is not easy to obtain efficient expression in the limb ectoderm (Suzuki et al., unpubl. data, 2008); the slow proliferation of ectoderm cells makes them relatively resistant to infection and subsequent integration by the viral genome. However, we can overexpress transgenes even in the ectoderm by applying electric pulses after dropping a DNA solution on the surface of the ectoderm (Kida et al., 2004). Finally, one can combine the electroporation and RCAS systems. For example, one can electroporate the RCAS retrovirus vector in the limb. Introduced vector can be integrated in the electroporated cells, resulting in the stable expression of transgenes and production of infective viral particles. When specific pathogen-free eggs are used, the virions produced infect surrounding cells, so that expansion of expression domain can be achieved. In addition, by electroporating a mixture of the RCAS and pCAGGS vectors, one can expect rapid but transient expression from pCAGGS at early stages and stable expression from RCAS at later stages.

By combining the two different methods, with electroporation as a common primary delivery system, one can control expression of transgenes both spatially and temporally. For example, we reported that *Tbx5* and *Tbx4* specify wing/leg identity in the chick limb bud, and that they are also necessary and sufficient for limb initiation (Takeuchi et al., 1999). In the chick embryo, the forelimb bud starts to develop from a restricted part of the LPM at the 15–20 somite level, whereas the hindlimb is formed at the 26–32 somite level at St. 14–16. Before limb bud initiation, both the limb fields and wing/leg identity are specified at the LPM. Therefore, to study limb initiation, we have to introduce a transgene before HH St. 14. Before St. 14, T-box transcription factor *Tbx5* is expressed only in the forelimb field, whereas *Tbx4* is only expressed in the hindlimb field. Takeuchi et al. electroporated RCAS *Tbx5-EGFP* or *Tbx4-EGFP* into future hindlimb or forelimb fields *vice versa*. They must be expressed continuously from initiation stages to later stages, because endogenous *Tbx4* and *Tbx5* are normally expressed throughout limb bud development. When *Tbx5-EGFP* was electroporated into the prospective hindlimb field, leg morphology completely changed. The scales were transformed to feathers, and the limb had three digits, as seen in the wing. In contrast, misexpression of *Tbx4-EGFP* in the prospective forelimb field induced the opposite transformation

of morphology. Feathers were converted to scales, and, like a leg, the limb had four digits. The phalange structure was also leg-like. The dramatic and revealing quality of these results makes it clear that the electroporation system is an excellent method to study specification of the wing/leg. Furthermore, when *Tbx5-EGFP* or *Tbx4-EGFP* in pCAGGS vector was electroporated at the future flank region, an extra wing or leg was formed, respectively (Takeuchi et al., 2003). Importantly, when *Tbx5* fused with a robust repressor domain from Engrailed (En-Tbx5) was misexpressed at the prospective forelimb field, a wing-less phenotype arose with a loss of a scapula bone (Takeuchi et al., 2003). In this experiment, RCAS En-Tbx5 was electroporated around HH St. 10, 16h before limb initiation starts. When the prospective limb fields were isolated at HH St. 9 and cultured for 24h, expression of *Tbx5* and *Tbx4* persisted in the isolated tissues (Saito et al., 2002). This result indicates that the prospective forelimb and hindlimb fields are specified before HH St. 9. Therefore, RCAS En-Tbx5 had to be electroporated as early as possible to inhibit the endogenous *Tbx5* function. At the moment, electroporation is the only method that fits the requirements of such experiments.

3.2 Important Reminders About Electroporation

As described above, electroporation is a powerful and excellent method for introducing transgenes into the limb bud and other tissues. But there are several precautions to consider in its use. First, we point out the physical damage induced by electric shocks during electroporation. When the voltage of the applied pulses is high or an electrode is attached to the LPM directly during electric pulses, truncated or malformed limb buds can arise at later stages. Although these artifacts arise only infrequently, it might be difficult to distinguish these artifactual phenotypes from those induced by the transgene. To minimize artifacts, one must be trained enough to obtain normal morphology after electroporation of an EGFP expression vector, which can be used as a negative control and a training plasmid. Second, it is difficult to electroporate an equal amount of DNA in each electroporation experiment. This means that electroporation is only suitable for qualitative analysis, and not quantitative assays.

3.3 New Applications for Electroporation in the Study of Limb Bud Development

Knockout mice are among the most important genetic tools for the study of gene functions, but mice lacking an essential gene may die before the appropriate stage for analysis. In these cases, electroporation provides an alternative method for the study of gene function even in mice.

One possible approach is knocking-down. An RCAS siRNA system was reported to be a useful tool in the study of limb bud development (Kawakami et al., 2003).

In these experiments, the authors inserted a pSuper siRNA promoter and an appropriately designed oligo into the RCAS vector to knock-down *MKP3* and clearly observed a cell death phenotype in the chick limb. If one can construct more effective RNAi vectors, it would be a useful tool even for mice, for which both *in utero* electroporation and *in vitro* embryo culture have recently been established (Tabata & Nakajima, 2001).

Another technique employing the CHAPOL retroviral library was used for analysis of cell lineages in the limb bud (Pearse et al., 2007). The members of this retroviral library each contain an alkaline phosphatase gene, as well as a 24 bp oligonucleotide of random sequence to mark each progenitor cell. After infecting the LPM with these replication incompetent retroviruses, the unique oligonucleotides of individual library members can be used as a tag to identify clonally related cells. Since we can electroporate DNA into a restricted area, it is possible to follow cell fates within a narrow area with this retroviral vector.

In order to be able to detect regional differences in gene activity within the limb bud, we developed an area-specific expression monitoring system. In the RCANBP vector, we inserted an exogenous SMAD1/5/8 responsive promoter to drive expression of the luciferase gene connected downstream. This vector enables us to monitor BMP signaling in the limb bud by measuring the enzymatic activity of luciferase (Suzuki et al., 2008). For normalization, the Renilla luciferase gene driven by the CMV promoter was used. At later stages, when each digit primordium is developing, a dual luciferase assay was performed with small tissue pieces isolated from the limb bud. Differential luciferase activities obtained by this method indicate that each digit primordium has different SMAD1/5/8 activities. This technique can be used for other signaling pathways by changing promoters that drive luciferase or other sensitive reporters.

Another innovative step is the application of electroporation to limb regeneration. It has been shown that nerves are necessary for limb regeneration (Kumar et al., 2007). When salamander limbs are amputated, stumps begin regeneration by forming a blastema. At this time point, regeneration does not occur if the nerves are removed from the limb. Kumar et al. reported that they could re-start limb regeneration even in denervated limbs by electroporating nAG into the blastema of the salamander. This result demonstrates that electroporation is an extraordinarily valuable new tool for the study of limb regeneration.

In the field of limb development, many of the most important genetic and embryological experiments resulted from new technical innovations. As described above, electroporation is one such innovative method. By combining electroporation with other newly developed techniques, new frontiers in modern experimental biology can be opened.

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