Sonic hedgehog is not a limb morphogen but acts as a trigger to specify all digits in mice

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INTRODUCTION

Sonic Hedgehog (Shh) is thought to be a prototypical vertebrate morphogen which can specify limb skeletal pattern. Understanding how Shh specifies limb skeletal pattern is integral to comprehending limb development, with potential applications in the field of regenerative medicine. Shh is secreted by posterior limb bud mesoderm cells, defined functionally as the "zone of polarising activity" (ZPA). Shh/ZPA controls the specification of all digits except the thumb, which is thought to form independent of Shh. Yet, how Shh specifies distinct digit identities still remains controversial. Previous chick studies suggest that changes in duration or concentration of Shh can change digit identity and number. In this paper, a genetic strategy was used to uncouple the Shh requirement in digit patterning from expansion or cell survival and test how Shh acts to specify digit pattern. It is suggested that Shh is required early for patterning but over a prolonged time, promotes survival and expansion.

METHODOLOGY

1. Manipulating genes to remove the function of Shh at controlled time intervals

Cre recombinase was used to insert a "stop" sequence between the promoter and Shh, causing the resultant mRNA sequence to have the Shh gene deleted, removing is expression.

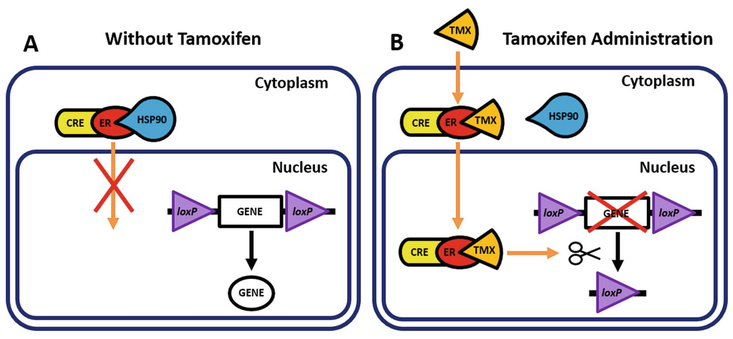


Figure 1: Manipulation of Shh expression with Tamoxifen[1]

2. Forcing cell survival in the absence of Shh

Previous studies have proved Shh's role in limb bud cell survival. The absence Shh induces apoptotic cell death. Thus, the pro-apoptotic genes, Bax/Bak, were also deleted in cells that had Shh knocked-out. These were referred to as Bax-CKO (conditional knock-out). Without these pro-apoptotic genes, it ensured cell survival.

3. X-gal staining

X-gal staining was used to map limb bud development in the different trials (with Shh being knocked-out at different time intervals), to determine the role of Shh in digit specification.

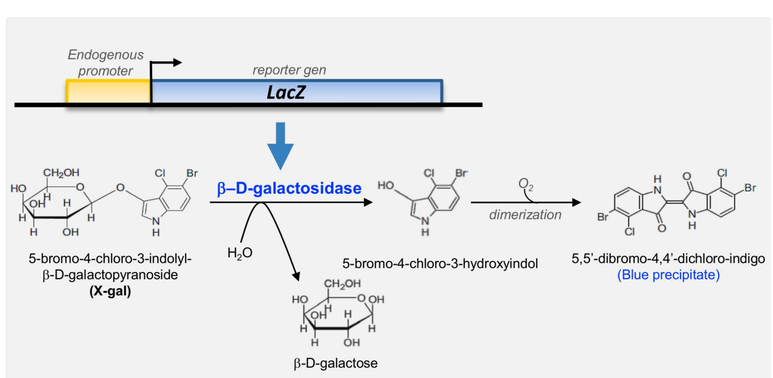


Figure 2: Role of LacZ gene in X-Gal staining[2]

The LacZ gene was first knocked-in into the mouse DNA at the Rosa26 locus, and activated only when Cre removes its inhibitory sequence. Upon administration of Tamoxifen, Cre-ER removes the inhibition of LacZ, thus allowing the β-galactosidase enzyme to be translated.

The β-galactosidase enzyme hydrolyses X-gal, the products of which oxidizes to form 5,5'-dibromo-4,4'-dichloro-indigo, which can then be stained blue, allowing the developmental pattern of limb buds to be studied.

RESULTS

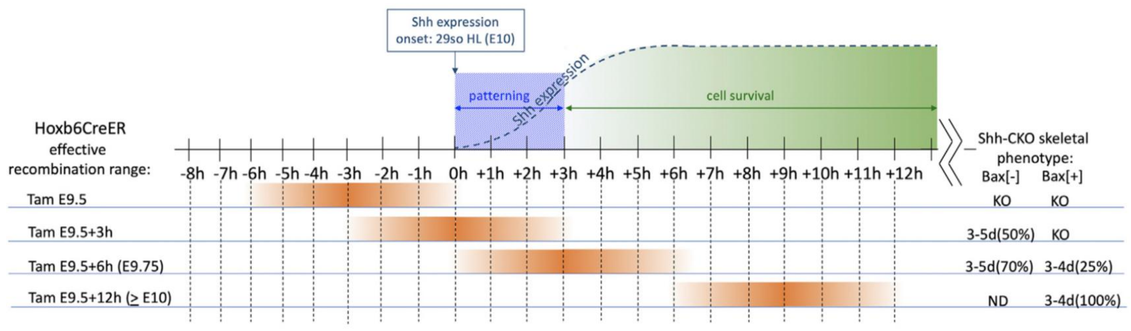


Figure 3: Summary of recombination timelines for tamoxifen (Tam)-induced Shh deletion at different times relative to Shh expression onset and subsequent digit outcomes. Tamoxifen timing was optimized to E9.5 + 3 after Shh expression onset but before it acts to maintain cell survival.

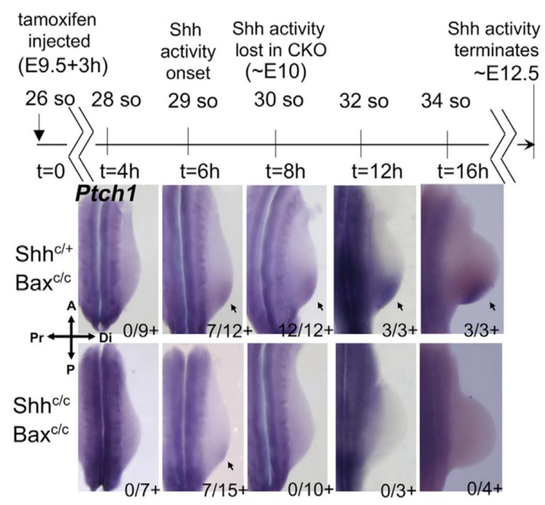


Figure 4: Shh activity assayed by Ptch1 RNA at times after Tam induction. Shh activity is first detectable at t=6h and non-detectable by t=8h in Shh-CKO embryos.

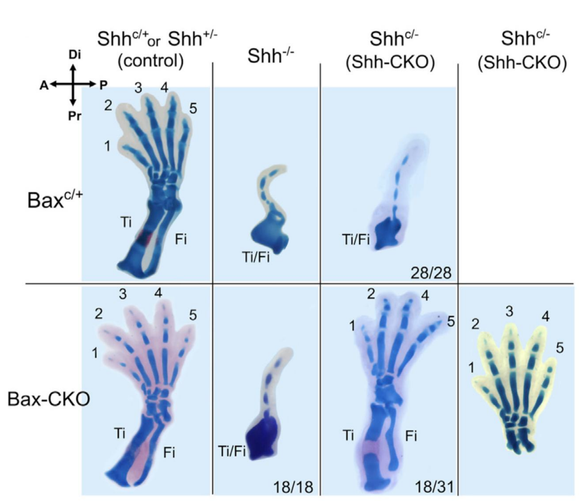


Figure 5: Skeletal staining (E16.5) after Bax/Bak and Shh removal by Tam (treated at E9.5 + 3 h). An early, transient Shh pulse is necessary and sufficient for normal digit and long bone formation with Bax/Bak removal.

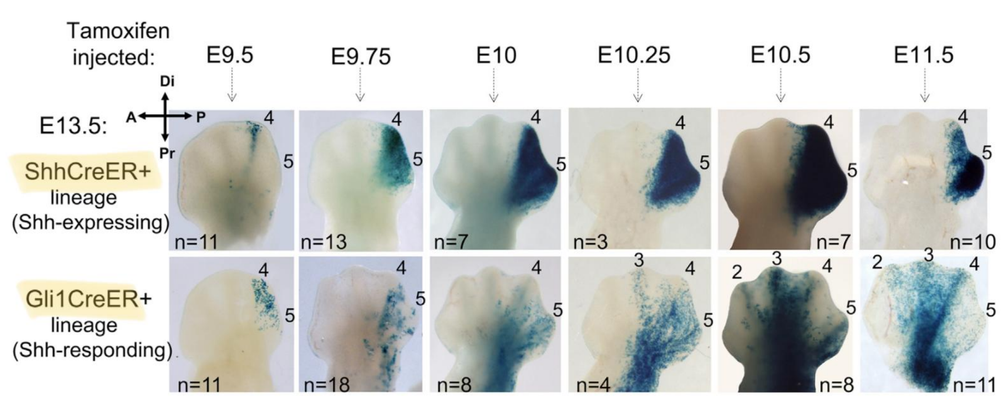


Figure 6: Distribution of RosaLacZ reporter+ descendants (in E13.5 digit rays) after single-dose tamoxifen given at time indicated. The direct action of Shh is very short range at early times after expression onset. Other digit progenitors that do not respond directly to Shh during this time window must be specified by an indirect mechanism

DISCUSSION

Summary

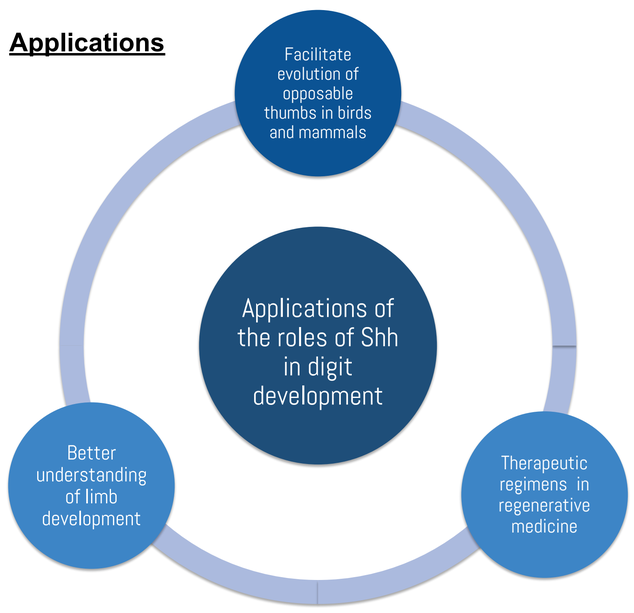
Transient, early Shh expression is essential to specify all digits and sustained expression is required to promote cell survival. Shh is not a limb morphogen but acts via an indirect mechanism to specify the non-ZPA digits.

Limitations

Shh-response reporters may have failed to detect low-level, transient longer-range activity.

Definitive demonstration that Shh acts indirectly to pattern limb digits will require identifying the factors involved.

Applications



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