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# Experimental Evidence That Preaxial Polydactyly And Forearm Radial Deficiencies May Share A Common Developmental Origin

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| **Corresponding Author:** | Wee Leon Lam, MB ChB, M.Phil, FRCS (Plast)<br>Royal Hospital for Sick Children<br>Edinburgh, UNITED KINGDOM |
| **Corresponding Author Secondary Information:** | |
| **Corresponding Author's Institution:** | Royal Hospital for Sick Children |
| **Corresponding Author's Secondary Institution:** | |
| **First Author:** | Wee Leon Lam, MB ChB, M.Phil, FRCS (Plast) |
| **First Author Secondary Information:** | |
| **Order of Authors:** | Wee Leon Lam, MB ChB, M.Phil, FRCS (Plast) |
| | Julia Dong Hwa Oh, BSc (Hons), MB ChB |
| | Edward J Johnson, BSc (Hons), PhD |
| | Sandra Poyatos Pertinez |
| | Chloe Stephens, BSc (Hons) |
| | Megan Gwyneth Davey, BSc (Hons), PhD |
| **Order of Authors Secondary Information:** | |
| **Abstract:** | Preaxial polydactyly is a congenital hand anomaly predominantly of sporadic occurrence, which is frequently associated with abnormalities of the Sonic hedgehog signalling pathway. In experimentally induced preaxial polydactyly, radial aplasia is also frequently observed. To determine if there is a correlation between preaxial polydactyly and radial aplasia, we induced ectopic Sonic hedgehog signalling during chicken limb development with application of a SMO-agonist or retinoic acid. Application of SMO-agonist caused malformations in 71% limbs including preaxial polydactyly (62%) and forearm abnormalities (43%). Retinoic acid application induced malformations in 56% of limb including preaxial polydactyly (45%) and forearm abnormalities (50%). Radial dysplasia and ulnar dimelia were observed in both experimental conditions. We demonstrate that ectopic Sonic hedgehog signalling may cause both preaxial polydactyly and predictable forearm anomalies and that these conditions could potentially be classified as one embryological group. We propose a unifying model based on known models of ectopic Sonic hedgehog signalling. |

# Experimental evidence that preaxial polydactyly and forearm radial deficiencies may share a common developmental origin

Wee Leon Lam[1], Julia Dong Oh[1, 2], Edward J Johnson[2], Sandra Poyatos Pertinez[2], Chloe Stephens[2], Megan G Davey[2]

1. Royal Hospital for Sick Children, Edinburgh, Scotland, UK
2. Division of Developmental Biology, The Roslin Institute, University of Edinburgh, Scotland, UK. In collaboration with Roslin Institute Chicken Embryology (RICE).

**Corresponding Author:**

Megan G Davey

Division of Developmental Biology,

The Roslin Institute,

University of Edinburgh,

Easter Bush, EH25 9RG,

Scotland, UK.

Email: megan.davey@roslin.ed.ac.uk

**Keywords:** Preaxial polydactyly; radial aplasia; retinoic acid; Sonic Hedgehog; OMT Classification

**Abstract**

Preaxial polydactyly is a congenital hand anomaly predominantly of sporadic occurrence, which is frequently associated with abnormalities of the Sonic hedgehog signalling pathway. In experimentally induced preaxial polydactyly, radial aplasia is also frequently observed. To determine if there is a correlation between preaxial polydactyly and radial aplasia, we induced ectopic Sonic hedgehog signalling during chicken limb development with application of a SMO-agonist or retinoic acid. Application of SMO-agonist caused malformations in 71% limbs including preaxial polydactyly (62%) and forearm abnormalities (43%). Retinoic acid application induced malformations in 56% of limb including preaxial polydactyly (45%) and forearm abnormalities (50%). Radial dysplasia and ulnar dimelia were observed in both experimental conditions. We demonstrate that ectopic Sonic hedgehog signalling may cause both preaxial polydactyly and predictable forearm anomalies and that these conditions could potentially be classified as one embryological group. We propose a unifying model based on known models of ectopic Sonic hedgehog signalling.

## INTRODUCTION

Pre-axial polydactyly (PPD) is a common congenital hand anomaly predominantly of sporadic occurrence. Wassel (1969) proposed a radiological classification for PPD conditions which has since been modified numerous times (Buck-Gramcko, 1998; Wood, 1978), most recently by Zuidam et al. (2008). These continuing efforts to 'classify' complex PPD conditions may reflect a lack of understanding into the underlying disease process.

Our current understanding remains that PPD is frequently caused by mutations which result in either the ectopic expression or activation of the Sonic hedgehog (SHH) signalling pathway (Anderson et al., 2012); a pathway that acts during embryonic limb development

(Riddle et al., 1993) to specify both digit number and anterior-posterior (radial-ulnar) digit identity (Tickle and Towers, 2017). Examples of mutations include alterations in the long-range limb specific enhancer of SHH (the zone of polarising activity regulatory sequence or ZRS) where altered anatomies are confined to the limb (Anderson et al., 2012), more systemic features such as Greig syndrome (OMIM #175700) or ciliopathic conditions such as Short rib polydactyly syndrome (Huber and Cormier-Daire, 2012; OMIM #616546). SHH signalling acts to induce formation of GLI-Activator proteins over GLI-Repressor proteins and in many syndromic conditions associated with PPD, activity of the GLI transcription factors downstream of SHH signalling are altered (Vortkamp et al. 1991; Davey et al., 2006, Tickle and Towers 2017). Experimental manipulations of SHH signalling in animal models have shown that SHH acts in a time and dose dependent manner (Tickle, 1981). Thus, it is predicted that PPD severity in humans could be due both to the strength and duration of ectopic SHH expression in the developing limb bud. The developmental stage at which SHH is ectopically expressed also influences the severity of PPD; ectopic SHH expression in early limb buds creates more severe defects than expression in more mature and patterned limb buds (Yang et al., 1997; Dunn et al., 2011).

Other than the autopod (hand), the ulna is also dependent on the SHH pathway for normal development. The ulna is partially derived from SHH expressing cells (Harfe et al., 2004) and in mice and chickens where SHH expression is depleted, the ulna is lost while the radius is maintained (Pagan et al., 1996; Chiang et al., 2001, Ros et al., 2003). Precisely-timed genetic disruption of SHH-signalling demonstrated that the ulna requires early/high levels of SHH signalling, equivalent to that of the most posterior digit, in order to develop (Zhu et al., 2008). In addition, strong prolonged ectopic anterior SHH signalling can cause ulnar dimelia (duplication) (Duprez et al., 1999, Yang et al., 1997). More commonly, however, radial

aplasia without dimelia is observed in conjunction with induced PPD in experimental models, either through anterior application of SHH protein or retinoic acid (SHH agonist) (Tickle et al., 1975, Eichele et al., 1985) or through early deletion of GLI3 (Bowers et al. 2012). So far from these experiments, it would suggest that a spectrum of effects could be elicited by ectopic SHH signalling, resulting in PPD and radial aplasia/ulnar dimelia.

We suggest a possible unifying hypothesis based on our understanding of the criteria of the limb morphogen including developmental stage of action, time and strength of ectopic SHH signal to explain the various degrees of PPD, radial aplasia and ulnar dimelia.

## METHODS

### Source and incubation of chicken eggs

ISA Brown chicken lines were maintained at The Roslin Institute under UK Home Office licence. Fertilised chicken eggs were incubated at 38°C. Stage of embryonic development was determined by Hamburger and Hamilton (HH; 1951).

### Smoothened agonist (SAG) application and mechanism of action

1µg smoothened agonist (SAG, Calbiochem, Darmstadt, Germany) was diluted in water and applied in 5uL applications so that embryos received 1µg, 2.5µg or 5µg doses between stages 15-21HH (see Supplementary Data 3 for optimisation of SAG dose). SAG was injected directly onto the embryo within the amniotic membrane. For information on the action of SMO see Supplemental Data 1 and Supplemental Fig 1.

### Retinoic acid (RA) application

AG1-X2 ion exchange resin beads were loaded with all-trans-retinoic acid as per Johnson et al (2014). Retinoic acid beads were implanted into a slit created under the base of the anterior distal AER of the limb bud at stages 17-21HH. This application of RA in this location is known to induce SHH in a precise and repeatable way in the limb mesenchyme (Riddle et al., 1993; Johnson et al., 2014).

### SHH protein application

Affi-Gel Blue Beads were soaked in SHH protein (Recombinant N-Terminus Mouse SHH protein; 461-SHH RandD Systems) and applied as per Tiecke and Tickle (2007).

### Skeletal staining and anatomy

Embryos were incubated at 38°C, fixed at E10 (RA and SAG manipulations) or E12 (SHH bead manipulations) in 5% TCA, stained overnight in 0.1% Alcian green in 70% acid alcohol

and dehydrated in graded steps to 100% ethanol and cleared in methyl salicylate. Anatomy is described as per Towers et al. (2011) for digits and Botelho et al. (2014) for carpals. For further details of bird limb anatomy see online Supplemental Data 2.

## RESULTS

In preliminary experiments using SAG applied to the developing limb we observed 4/76 independent incidences of loss or reduction of the radius, 1 of which was associated with very mild preaxial polydactyly (see Supplemental Data 3). As a loss of the radius in association with PPD has also been previously demonstrated, although not well described, in classical experiments in which PPD has been induced through application of posterior limb mesenchyme expressing *SHH*, *SHH* expressing fibroblasts or retinoic acid (RA) to the anterior wing bud (Tickle 1981; Eichele et al. 1985, Yang et al. 1997), we therefore set out to examine if a loss of the radius could be directly associated with induction of PPD upon abnormal activation of SHH signaling. We undertook two approaches; application of 2.5ug SAG to stage 16HH to induce widespread SHH-related signaling (Group 1) or application of RA to the anterior limb bud at stages 17-21HH to induce localized ectopic SHH signaling (Group 2).

### Group 1

Group 1 exhibited limb malformations in the majority of embryos with a high incidence of preaxial polydactyly in affected limbs (Fig. 1K-M) (Table 1). Of the 24 affected limbs with altered zeugopod (forearm) patterning, the radius demonstrated some degree of 'ulnarisation' (Fig. 1K, L). Examples of altered anatomy included alteration of the proximal radial head to resemble incomplete olecranon development around the elbow joint ('o'; Fig. 1K), shortening and thickening of the radius and widening of the distal head of the radius (Fig. 1K, L). We also observed loss of the radius (Fig. 1M) associated with PPD. In humans, first digit ('thumb', SHH-independent) hypoplasia is typically observed with radial aplasia; in birds, however, digit 1 is a SHH dependent digit (Towers et al., 2008) and would not be predictably removed by additional SHH signaling. There were no instances of ulnar dimelia in Group1 specimens (Table 1).

## Group 2

In Group 2, following RA application to the anterior limb bud, the majority of limbs again exhibited limb malformations (Fig. 1B-I; Table 1). The incidence of PPD in the autopod was similar to the SAG treated embryos, but there was a much higher percentage of forearm involvement including radial aplasia or hypoplasia with or without associated PPD (Fig. 1F, G, H, I). In addition, there were seven cases of true ulnar dimelia with the anterior forearm bone displaying both an olecranon proximally and a loss of the chicken radial carpal bone (radialus) distally (Fig. 1E, Supplemental data 2). Thus, utilizing two methods for activation of SHH signaling in the limb, we have shown consistently that the zeugopod, as well as the autopod is affected if SHH signaling is ectopically activated.

## Stage and Location Dependence of RA Effect on Polydactyly

Both the stage of embryonic development and the precise location of RA beads can lead to variation in the extent of PPD that is induced (Eichele et al., 1985). To determine if the embryonic stage of RA application and location of RA also influenced the outcomes of forelimb malformations, we re-analysed our data both using subsets of samples in which the embryonic stage of manipulations were precisely recorded (n=31) and in which we could locate and map the final location of the bead within the E10 limb as an indication of initial bead placement (n=23). While limb truncations (2/30) including transverse deficiency and digit truncations were only observed at the earliest time point (stage 17HH), PPD of both 'mild' forms (one extra digit) and severe forms (mirror hand) were observed at both early and latter stages: 19HH (2 mild and 1 severe) and 20HH (3 mild and 2 severe) while stage 21HH exhibited only severe PPD (2/2 atypical mirror hand). Radial deficiency, however, was also observed at all stages: 17HH, 19HH, 20HH and 21HH (n=7) associated with no PPD (2/7),

mild PPD (2/7) and severe PPD (3/7). The location of bead placement also played an important role in alteration of limb anatomy and beads which were centered on the shoulder joint were more likely to result in a change in limb anatomy (red locations gave rise to limb malformations) (Fig. 1J).

**SHH protein manipulation of limb bud**

Finally, to demonstrate that manipulation of the developing limb bud with SAG or RA is comparable to manipulation of SHH signaling, we directly manipulated anterior developing limb buds at stage 20HH with recombinant SHH protein. Surviving embryos showed induction of mild PPD (n=2) (Fig. 1P, Q) and associated alterations to the radius, including distal thickening and shortening of the radius (n=1). Transformation to an ulna (n=1) included loss of the anterior radialus (ra; c.f. Fig. 1O with 1Q). The radial-ulna transformation was akin to the morphology observed in SAG and RA application. With SHH protein application we did not observe a loss of the radius although this has been previously reported (Yang et al., 1997).

## DISCUSSION

PPD, radial aplasia and ulnar dimelia are seen clinically as separate entities not only because of differences in location of aberrant anatomy but also their different treatment regimes. Our results have suggested that these conditions could be points on a continuum of aberrations caused by abnormal SHH signaling pathway induction in the embryonic arm, similar to a hypothesis first proposed by Al-Qattan (2013).

In human, mouse and chicken limbs, digit identity is thought to be determined by the SHH pathway. According to the Growth/Specification Model (Towers et al. 2008) and based on the French Flag hypothesis (Wolpert 1968), digit number and identity has been shown to be dependent on the strength and duration of normal SHH signaling. These hypotheses also predict that a spectrum of PPD defects would be induced by differences in strength and length of ectopic SHH expression. As SHH signaling acts also during zeugopod development, we hypothesise that ectopic SHH expression may also lead to forearm pathologies. Despite the numerous studies focusing on digits, relatively little attention has been on zeugopod formation and abnormal phenotypes caused by aberrations in the SHH signaling pathway.

In our stage compared experiments, interpretation of results is complex but some causes of variation may be hypothesised. Early limb buds exhibited either severe truncation defects or mild PPD. The truncations are similar to X-radiation induced cell death in the chicken limb with both anterior and posterior axes equally effected (Galloway, 2009). This suggests that young limb buds (stage 19HH) are either more vulnerable to cell death caused by RA application or that the distal limb elements have been re-specified (Roselló-Díez et al., 2011) as RA specifies proximal patterning. Early application did not induce strong PPD; possibly as the stage 19HH anterior limb bud may not be competent to express strong SHH, i.e. the

206 ‘priming phase’ maybe longer (Eichele et al., 1985). On the other hand, application of RA to
207 stage 21HH (latter stage) induced PPD in a manner that was comparable to a mirror-hand
208 (Eichele et al., 1985), a phenotype considered inducible only by the strongest ectopic SHH
209 signal (Yang et al., 1997). At stage 20HH, induced PPD phenotypes were of an intermediate
210 type as compared to those induced between 19HH and 21HH, demonstrating that there is a
211 continuum based on ectopic SHH expression.
212
213 While the severity of PPD could be correlated over time (early, intermediate and late), a
214 reduction of the radius was seen in all stages. This suggests that the time during which the
215 radius is susceptible to SHH signaling is both earlier and more prolonged than the digits. This
216 is in agreement with the Summerbell hypothesis that the zeugopod is patterned before the
217 autopod (Summerbell, 1974, López et al., 1995) or that the chondrogenic precursors of the
218 radius are vulnerable to changes in signaling before digit precursors (Galloway et al., 2009).
219 Our results are consistent with mouse studies in which an early loss of GLI-Repressor activity
220 causes tibial loss in conjunction with PPD but later timed deletions only cause PPD (Bowers
221 et al., 2012). In addition, our results also suggest that radial aplasia/hypoplasia can be induced
222 by a wide range of strength in SHH signaling, although ulnar dimelia was only induced in
223 limbs manipulated directly with SHH/RA and not with SAG (weaker agonist). The RA-
224 induced PPDs were the most extreme, where anterior digits of the ‘index type’ were
225 completely lost. As RA is known to induce a large area of anterior ectopic SHH signaling,
226 this suggests that dimelia only occurs when there are high ectopic levels of SHH signaling.
227 Indeed, application of SHH only caused a brief but strong activation of the SHH pathway.
228 With this brief activation, we were able to produce only a forelimb phenotype (dimelia),
229 confirming that the radius is susceptible to SHH signaling at an earlier time point. Finally,
230 dimelia was only induced by RA/SHH in manipulations of older limb buds at stage 20HH+,

showing that the conversion of identity is a later occurrence than specification of an anterior zeugopod element.

We therefore suggest that there are two time-dependent phases in the induction of the zeugopod pattern (Fig. 2). Phase 1 is dependent on low concentrations of SHH and during this time period, the zeugopod tissues become 'primed' to form two zeugopod elements with no set identity. Additional anterior SHH signaling during this phase ablates first the presumptive anterior zeugopod element and at higher levels, the posterior zeugopod element, resulting in a limb truncation. This illustrates that even when they are first established, the presumptive radius and ulna have different developmental potentials and sensitivity to SHH signaling. If not exposed to additional SHH signaling, both zeugopod elements would differentiate into a 'default' radius as is observed in the RAZ mouse in which lowered SHH signaling results in two radii (Krebs et al. 2003). During Phase 2, additional SHH signaling does not ablate the radius but instead acts to transforms it into an ulna. We suggest this is a prolonged developmental phase, illustrated by the partial 'ulna transformations' in which the radius becomes shorter and proximally retains a radius articulation with the elbow but distally becomes ulna-like. Both phases can overlap with the period of digit patterning, although our evidence suggests Phase 1 occurs slightly earlier than digit patterning.

A classification system of human PPD based on strength and timing of ectopic SHH can therefore be proposed based on our work and that of others. For example, our results coincided with the hypothesis of Al-Qattan (2013) that minor to moderate ectopic SHH signaling results in PPD and its variants. Furthermore, we conclude that radial dysplasia and ulnar dimelia can occur in conjunction with ectopic SHH signaling and are likely to be associated with higher and earlier expression of ectopic SHH. Our main conclusion remains,

however, that PPD, radial aplasia and ulnar dimelia are part of a single disease spectrum likely involving ectopic SHH activities.

If these various conditions were observed simply from a clinician standpoint, the conclusion that they belong to a similar spectrum of disease progression may never have been reached. As such, our study also serves to highlight the importance of collaboration between surgeons and developmental biologists; a collaboration that had been recently emphasised with the international replacement of the Swanson classification by the Oberg, Manske and Tonkin (OMT) (Oberg et al., 2010) classification. The OMT system invite 3-yearly reviews and invite contributions as a result of new knowledge gained from experimental studies. Our study may therefore potentially contribute to the OMT system in a number of ways: (1) in the OMT, conditions are separated into IA (forearm) and IB (hand plate), suggesting that the conditions of PPD, radial aplasia or dimelia occur as an 'either-or' phenomenon. Our results would re-emphasise the importance of viewing these conditions as an embryological continuum and a spectrum of related disorders; (2) the description of radial aplasia in IA2i only allows for the association of thumb hypoplasia, not PPD or multiple fingers. It may be worthwhile remembering that PPD can occur with radial aplasia (Marangoz and Leblebicioğu, 2006; Yildirim et al., 2005; Al-Qattan et al., 1998; Al-Qattan, 2012); and (3) the conditions of radial polydactyly and triphalangeal thumb are classified separately under IB2iii and IB2iv respectively (Table 2) to highlight they are different clinical conditions but the path-embryological process is less distinct.

In summary, we found experimentally that PPD, radial dysplasia and ulnar dimelia can be part of a single spectrum involving aberrations in the SHH pathway. A unifying theory of

disease pathogenesis may inform the hand surgeon about surgical anatomy and lead to new avenues of research.

## Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.


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## REFERENCES


Al-Qattan MM, Al-Thunayan A, De Cordier M et al. Classification of the mirror hand-multiple hand spectrum. J Hand Surg Br. 1998, 23: 534-6.

Al-Qattan MM, Al Abdulkareem I, Al Haidan Y et al. A novel mutation in the *SHH* long-range regulator (ZRS) is associated with preaxial polydactyly, triphalangeal thumb, and severe radial ray deficiency. Am J Med Genet. 2012, 158A: 2610–2615.

Al-Qattan MM. Preaxial polydactyly of the upper limb viewed as a spectrum of severity of embryonic events. Ann Plast Surg. 2013, 71: 118-24.

Anderson E, Peluso S, Lettice LA et al. Human limb abnormalities caused by disruption of hedgehog signaling. Trends Genet. 2012, 28:364-73.

Botelho JF, Ossa-Fuentes L, Soto-Acuña S et al. New developmental evidence clarifies the evolution of wrist bones in the dinosaur-bird transition. PLoS Biol. 2014, 12: e1001957

Bowers M, Eng L, Lao Z, Turnbull RK, Bao X, Riedel E, Mackem S, Joyner AL. Limb anterior-posterior polarity integrates activator and repressor functions of GLI2 as well as GLI3. Dev Biol. 2012, 370(1):110-24.

Buck-Gramcko D. Triphalangeal thumb. In: Buck-Gramcko D, ed. Congenital malformation of the hand and forearm. Edinburgh, Churchill Livingstone, 1998, 403-424.

Chiang C, Litingtung Y, Harris MP et al. Manifestation of the limb prepattern: limb development in the absence of sonic hedgehog function. Dev Biol. 2001, 236: 421-35.

Davey MG, Paton IR, Yin Y et al. The chicken talpid3 gene encodes a novel protein essential for Hedgehog signaling. Genes Dev. 2006, 20: 1365-77.

Dunn IC, Paton IR, Clelland AK et al. The chicken polydactyly (Po) locus causes allelic imbalance and ectopic expression of Shh during limb development. Dev Dyn. 2011, 240: 1163-72.

Duprez D, Lapointe F, Edom-Vovard F et al. Sonic hedgehog (SHH) specifies muscle pattern at tissue and cellular chick level, in the chick limb bud. Mech Dev. 1999, 82: 151-63.

Eichele G, Tickle C, Alberts BM. Studies on the mechanism of retinoid-induced pattern duplications in the early chick limb bud: temporal and spatial aspects. J Cell Biol. 1985, 101: 1913-20.

Galloway JL, Delgado I, Ros MA et al. A reevaluation of X-irradiation-induced phocomelia and proximodistal limb patterning. Nature 2009, 460: 400-4.

Hamburger V and Hamilton HL. A series of normal stages in the development of the chick embryo. Dev Dyn. 1951, 195: 231-72.

330 Harfe BD, Scherz PJ, Nissim S et al. Evidence for an expansion-based temporal Shh gradient
331 in specifying vertebrate digit identities. Cell. 2004, 118: 517-28.
332 Huber C1, Cormier-Daire V. Ciliary disorder of the skeleton. Am J Med Genet C Semin Med
333 Genet. 2012, 160: 165-74.
334 Johnson EJ, Neely DM, Dunn IC et al. Direct functional consequences of ZRS enhancer
335 mutation combine with secondary long range SHH signalling effects to cause preaxial
336 polydactyly. Dev Biol. 2014, 392: 209-20.
337 Krebs O, Schreiner CM, Scott WJ Jr et al. Replicated anterior zeugopod (raz): a
338 polydactylous mouse mutant with lowered Shh signaling in the limb bud. Development 2003,
339 130: 6037-47.
340 López SL, Dono R, Zeller R et al. Differential effects of retinoic acid and a retinoid
341 antagonist on the spatial distribution of the homeoprotein Hoxb-7 in vertebrate embryos. Dev
342 Dyn. 1995, 204: 457-71.
343 Marangoz S and Leblebicioğlu G. Thumb Polydactyly With Radius Hypoplasia—A Case
344 Report. J Hand Surg Am. 2006, 31: 1667-1670.
345 Oberg KC, Feenstra JM, Manske PR et al. Developmental biology and classification of
346 congenital anomalies of the hand and upper extremity. J Hand Surg. 2010, 35: 2066-76.
347 Pagan SM, Ros MA, Tabin C et al. Surgical removal of limb bud Sonic hedgehog results in
348 posterior skeletal defects. Dev Biol. 1996, 180: 35-40.
349 Riddle RD, Johnson RL, Laufer E et al. Sonic hedgehog mediates the polarizing activity of
350 the ZPA. Cell. 1993, 75: 1401-16.
351 Ros MA, Dahn RD, Fernandez-Teran M et al. The chick oligozeugodactyly (ozd) mutant
352 lacks sonic hedgehog function in the limb. Development. 2003, 130: 527-37.
353 Roselló-Díez A, Ros MA, Torres M. Diffusible signals, not autonomous mechanisms,
354 determine the main proximodistal limb subdivision. Science. 2011, 27;332: 1086-8.

Summerbell D. Interaction between the proximo-distal and antero-posterior co-ordinates of positional value during the specification of positional information in the early development of the chick limb-bud. J Embryol Exp Morphol. 1974, 32: 227-37.

Tickle C. The number of polarizing region cells required to specify additional digits in the developing chick wing. Nature. 1981, 289: 295-8.

Tickle C, Summerbell D, Wolpert L. Positional signalling and specification of digits in chick limb morphogenesis. Nature. 1975, 254:199-202.

Tickle C and Towers M. Sonic Hedgehog Signaling in Limb Development. Front Cell Dev Biol. 2017, 28;5: 14.

Tiecke E and Tickle C. Application of sonic hedgehog to the developing chick limb. Methods in molecular biology. 2007, 397: 23-33.

Towers M, Mahood R, Yin Y et al. Integration of growth and specification in chick wing digit-patterning. Nature. 2008, 452: 882-6.

Towers M, Signolet J, Sherman A et al. Insights into bird wing evolution and digit specification from polarizing region fate maps. Nat Commun. 2011, 2: 426.

Vortkamp A, Gessler M., Grzeschik K-H. GLI3 zinc-finger gene interrupted by translocations in Greig syndrome families. Nature 1991, 352: 539-540.

Wassel HD. The results of surgery for polydactyly of the thumb. A review. Clin Orthop Relat Res. 1969, 64: 175-93.

Wolpert L. The French Flag problem: A contribution to the discussion on pattern development and regulation. Towards a theoretical biology. 1968, 1: 125-33.

Wood VE. Polydactyly and the triphalangeal thumb. J Hand Surg Am. 1978, 3: 436–44.

Yang Y, Drossopoulou G, Chuang PT, et al. Relationship between dose, distance and time in sonic hedgehog-mediated regulation of anteroposterior polarity in the chick limb. Development. 1997, 124: 4393-404.

Yildirim S, Taylan G, Aydogdu E et al. The true triplication of the thumb: a case of unclassified thumb polydactyly. Ann Plast Surg. 2005, 55: 321-323.

Zhu J1, Nakamura E, Nguyen MT et al. Uncoupling Sonic hedgehog control of pattern and expansion of the developing limb bud. Dev Cell. 2008, 14: 624-32.

Zuidam JM, Selles RW, Ananta M et al. A classification system of radial polydactyly: Inclusion of triphalangeal thumb and triplication. J Hand Surg Am. 2008, 33: 373-7.

## FIGURE LEGENDS

**Figure 1 - Radial aplasia is observed within the spectrum of ectopic SHH signalling induced limb malformations**

**B-Q** Examples of limbs in which ectopic SHH signalling had been induced with through Retinoic Acid (RA) **B-J** Application of SAG (**K-M**) or application of SHH protein (**P,Q**). Limbs induced by retinoic acid were categorised by observation of the polydactylous phenotype, i.e. 'Mild PPD', 'Severe PPD', Severe PPD (Mirror Hand-like), Dimelia. In all categories limbs with radial aplasia/dysplasia (**F-I, M**) or limbs with a transformation from radius-ulna were observed (partial distal ulna; **M**), except in Dimelia (**E**). **J.** Limb with a recording of location of RA bead and whether it caused limb malformation (red). Control limbs **A, N, O**. Abbreviations =H- humerus, r- radius, u- ulna, 1- digit 1, 2- digit 2, 3- digit 3, o- olecranon, ra/it- radialus/intermedium, it- intermedium, pi- pisiform, b- blip, slc- . Changes in anatomy are markers in red annotation. *- missing bony element.

**Figure 2 - Hypothesised Correlation between Longitudinal Deficiencies and SHH Signalling.**

**A.** We propose, based on our experimental evidence and that of others, that the strength of ectopic SHH signalling causes a spectrum of forelimb malformations, which radial-ulna transformations at lower levels of ectopic SHH signalling and dimelia as a result of high ectopic SHH signalling. **B**. We further propose that limb malformations induced by ectopic SHH expression are determined both by the strength of SHH signalling and the time which they are expressed and that SHH may be an underlying cause of a variety of limb malformations not currently classed as related. Early ectopic SHH signalling can induce first transverse deficiency, then radial deficiency, then radius-ulna transformations and finally polydactyly. Depending on the length and strength of ectopic SHH signalling a number of different malformations can be induced which model human limb malformations. We propose radial longitudinal deficiency with PPD is theoretically part of this spectrum and would be due to early strong and prolonged ectopic SHH expression.

**SUPPLEMENTARY MATERIAL**

**Fig S1**

**Appendix S1: Supplementary Data 1, 2 & 3**

Figure 1



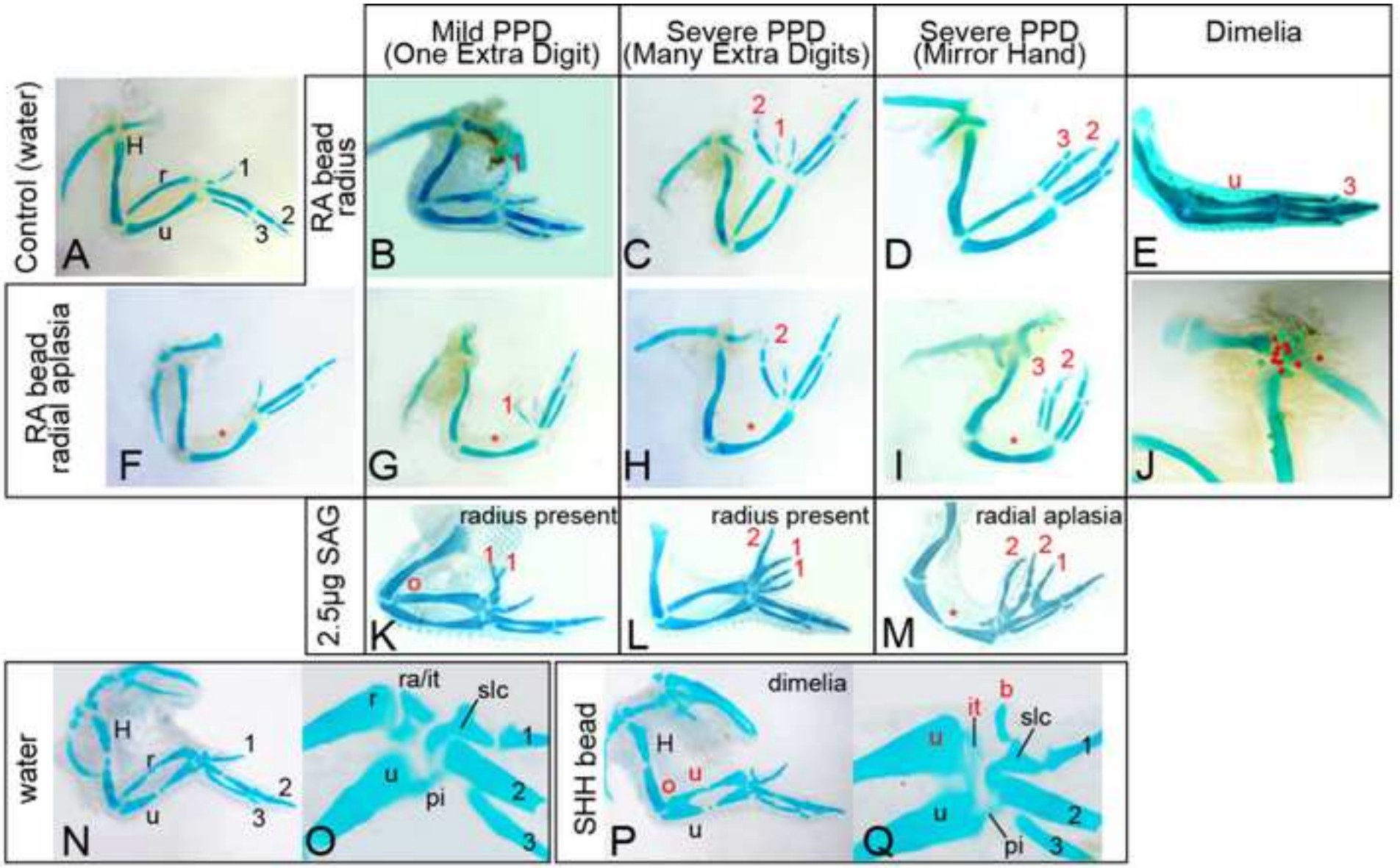

Figure 2



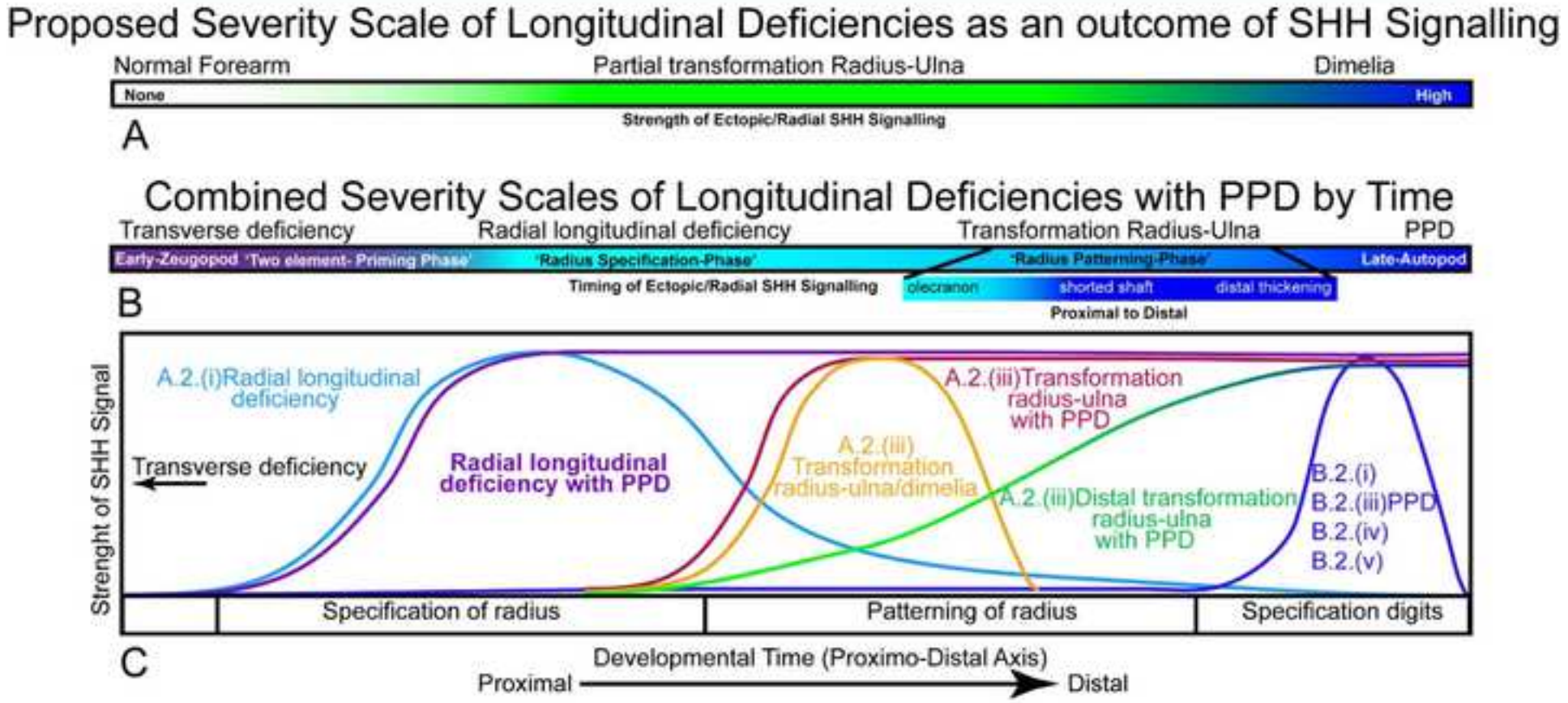

**Table 1: The number of Limbs with Manipulations and incidence**

| **Malformations** | **Group 1**<br>**SAG**<br>**(56 limbs)** | **Group 2**<br>**RA**<br>**(55 limbs)** | **SHH** |
|---|---|---|---|
| **Total limbs with malformations** | 40/56 (71%) | 31/55 (56%) | 2/2 (100%) |
| **Preaxial polydactyly** | 35/40 (87.5%) | 25/31 (80%) | 1/2 (50%) |
| **Forearm involvement** | 24/40 (60%) | 28/31 (90%) | 2/2 (100%) |
| **Transverse deficiency** | 0/24 (0%) | 1/28 (3.5%) | |
| **Ulnarisation of radius** | 4/24 (17%) | 0/28 (0%) | 1/2 (50%) |
| **Ulnarisation of radius *with PPD*** | 17/24 (71%) | 5/28 (18%) | 1/2 (50%) |
| **Ulnar dimelia *with mirror hand*** | 0/24 (0%) | 7/28 (45%) | |
| **Radial aplasia** | 0/24 (0%) | 4/28 (14%) | |
| **Radial hypoplasia *with missing digits*** | 0/24 (0%) | 1/28 (3.5%) | |
| **Radial aplasia *with PPD*** | 2/24 (8%) | 7/28 (45%) | |
| **Radial dysplasia *with mirror hand*** | 1/24 (4%) | 3/28 (11%) | |

**Table 2: Excerpt from the current OMT Classification of Congenital Anomalies of the Hand and Upper Limb (Oberg et al. 2010).**

| **I. MALFORMATIONS** | |
|---|---|
| **A. Abnormal axis formation/differentiation – entire upper limb** | B. Abnormal axis formation/differentiation – hand plate |
| **2. Radial-ulnar (anterior-posterior) axis** | 2. Radial-ulnar (anterior-posterior) axis |
| (i) **Radial longitudinal deficiency –Thumb hypoplasia (with proximal limb involvement)** | (i) Radial deficiency (thumb – no forearm/arm involvement) |
| (ii) **Ulnar longitudinal deficiency** | (ii) Ulnar deficiency (no forearm/arm involvement) |
| (iii) **Ulnar dimelia** | (iii) Radial polydactyly |
| (iv) **Radioulnar synostosis** | (iv) Triphalangeal thumb |
| (v) **Congenital dislocation of the radial head** | (v) Ulnar dimelia (mirror hand – no forearm/arm involvement) |
| (vi) **Humeroradial synostosis – Elbow ankyloses** | (vi) Ulnar polydactyly |
| (vii) **Madelung deformity** | |

**TITLE PAGE**

**Title:**

Experimental Evidence That Preaxial Polydactyly And Forearm Radial Deficiencies May Share A Common Developmental Origin

**Author names/degrees:**

1. W L Lam[1] MBChB, M Phil, FRCS (Plast)
2. J D Oh[1, 2], BSc (Hons), MBChB
3. E D Johnson[2], BSc (Hons), PhD
4. S Poyatos Pertinez[2]
5. C Stephens[2], BSc (Hons)
6. M G Davey[2], BSc (Hons), PhD

**Establishments:**

1. Royal Hospital for Sick Children, 9 Sciennes Road, EH9 1LF, Edinburgh, Scotland, UK
2. Division of Developmental Biology, The Roslin Institute, University of Edinburgh, Easter Bush, EH25 9RG, Scotland, UK. In collaboration with Roslin Institute Chicken Embryology (RICE).

**Corresponding Author:**

Megan G Davey; Division of Developmental Biology, The Roslin Institute, University of Edinburgh, Easter Bush, EH25 9RG, Scotland, UK. Email: megan.davey@roslin.ed.ac.uk, +44 0131 6519170

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