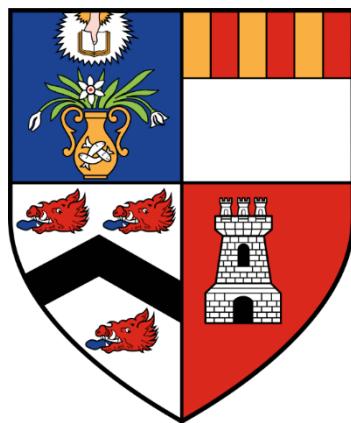


Undergraduate Essays



A collection of (exam) essays presented for the degree of Human Embryology and Developmental Biology, Bachelor of Science with Honours at the University of Aberdeen

Paul Oliver Shepherd

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What have genetically modified animals told us about the genetics and aetiology of the human lower limb abnormality, ‘clubfoot’?

Paul Shepherd

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‘Clubfoot’, or congenital talipes equinovarus (CTEV), is a lower limb defect that occurs during development. It is characterised by persistent plantar flexion and medial rotation of the foot (seen on Figure 1 in the appendix), which if left untreated leads to permanent disability. CTEV is among the most common paediatric orthopaedic conditions effecting 1-3 in every 1,000 live births (Lupo et al. 2019; Dobbs et al., 2012; Cartlidge, 1984). The majority of CTEV is idiopathic, meaning that clubfoot is the only problem in an otherwise healthy child, but it can also be associated with other syndromes including spina bifida (Wynne-Davies 1964). Clubfoot has been a part of human history through the ages, and the fate of individuals with clubfoot depended on the attitude of their society, ranging from casting aside to die at birth all the way to Gods (Ramachandran, 2006; Strach, 1986). Hippocrates (born approximately 460BC) established the foundations for modern clubfoot treatment urging repeated manipulation and fixation with strong bandages as soon as possible after birth (Strach, 1986). Today’s Ponseti method is not too dissimilar, which offers a low-cost treatment involving a treatment phase, including serial; manipulation, casting and tenotomy, and a maintenance phase, using foot abduction braces (Ponseti 1997). However, despite centuries for development this ‘modern’ method still results in 10-15 percent relapsing and the majority experiencing leg fatigue (Ippolito 2009). Human population genetics and twin studies, with identical twins having a higher concordance rate (at 33 percent) than fraternal twins (3 percent), strongly suggest a genetic basis for CTEV (Lupo et al., 2019; Miedzybrodzka, 2003), but there is an incomplete understanding of CTEV’s genetic factors and aetiology. This genetic basis can be seen with inbreeding probably increases the probability of clubfoot both human and horses (Comparini et al. 2019; Hawass et al., 2010). Genetically modified animals are a valuable research tool for knowing the causes and genes involved in CTEV through which we develop the possibility to future human treatment options. This essay will discuss the hypotheses for the causes of human clubfoot before looking at genetically modified animal models to explain the cause and genetics of human clubfoot, then it will finally touch on the future genetic based treatment options for clubfoot.

There are six hypotheses for the cause of human clubfoot with it long being hypothesised that ‘Clubfoot’ could be due to muscle weakness caused by malformations in the musculoskeletal and/or nervous system (Feldbrin et al., 1995; Yoshimura et al., 1988; Nonaka et al., 1986; Handelsman and Badalamente, 1981). The following have been established in humans and can be broken down into; (1) The bone/joint hypothesis, which is where bone abnormalities, specifically endochondral and perichondral ossification disruption, are the cause (Fritsch and Eggers 1999). (2) The connective tissue hypothesis, which is the belief that increased fascia, ligaments, tendon sheaths and fibrous tissue in muscle causing retracting fibrosis could be the cause (Ippolito and Ponseti 1980), but there is conflicting evidence with no observed abnormality in connective tissue in humans (Atlas et al., 1980). (3) The vascular hypothesis, which suggests that a reduction in perfusion, specifically through the anterior tibial artery, can cause calf muscle atrophy in humans (Merrill et al., 2011; Atlas et al., 1980). (4) The neurological hypothesis, which could be due to nerve abnormalities observed in 18 out of 44 idiopathic CTEV, 8 of which had abnormalities at the spinal level (Nadeem et al. 2007). The other two theories for the cause of human clubfoot that are more based on observation of traits are; (5) The ‘mechanical forces’ or ‘positional’ hypothesis, which suggests that it is the restriction of foetal foot movement in the uterus possibly due to reduced amniotic fluid volume (which on its own causes neurological problems) that causes clubfoot (Farrell et al., 1999; Hoffa 1902). (6) The failure to progress in development

hypothesis, which is just the idea that the foot in severe clubfoot resembles the embryonic foot two months into development (Kawashima and Uhthoff 1990; Böhm 1929). Now that we have established the hypothesised causes of human clubfoot we will focus on the genetically modified animals that have been studied to put forward evidence to actually define the cause with respect to the first four hypotheses. The bone/joint hypothesis was reproduced in animals using retinoic acid as a teratogen to cause induced foetal growth retardation and reduced ossification of the same hindlimb bones as in congenital clubfoot (Liu et al., 2010; Delgado-Baeza et al., 1999). But as it is teratogen induced, it is probably not as useful of a model as genetically modified peroneal muscular atrophy (pma) mutant mice whose skeletal changes were clearly seen to resemble human CTEV using micro-magnetic resonance imaging, the results of which can be seen in Table 1 and Figures 2 and 3 in the appendix (Duce et al., 2010). This evidence establishes, in this essay, that pma mice are a good model for human CTEV as the pma mice show the same persistent plantar flexion and medial rotation of the ankle and toes, as well as the mid-foot inversion and adduction. They also showed that it occurred from embryonic day 14.5 with the exception of the rotation which took longer, and therefore supports the failure to progress in development hypothesis, thus linking hypothesis 1 and hypothesis 6. This same study also showed that muscle volume of shank muscle was significantly reduced by approximately 70 percent, the specific muscles being the fibularis (peroneus) tertius, extensor digitorum longus, and extensor hallucis longus muscles, which make up the antero-lateral muscle group. This was slightly different to what has been seen to happen in humans which was mainly the posterior compartment muscles (Ippolito et al., 2009) as opposed to this antero-lateral muscle group observed in pma mice. So, although not entirely consistent with human CTEV they both the pma mice and humans showed that muscle changes are a key feature, and just for clarity this does not point to the connective tissue being the route course so doesn't support hypothesis 2. Ultimately this study showed numerous anatomical similarities (specifically in bone and muscle) between humans and pma mice.

The pma gene has been mapped to chromosome 5 in mice with the gene order being: centromere-D5Mit263-[2.65 cM]-D5Mit141-[2.56 cM]-pma-[5.13 cM]-D5Mit97-telomere in pma mouse (Katoh et al., 2003), opens up the possibility to fully understand the genetic basis and developmental mechanisms behind CTEV. Figure 4 in the appendix was taken from this paper and clearly shows the morphological characteristics of the twisted foot, the reduced muscle volume, previously discussed, and the underdeveloped peroneal nerve. A recent human clubfoot genome wide association study showed that no SNP reached genome-wide level significance, but the strongest evidence pointed to an intergenic SNP on chromosome 12q24.31 between NCOR2 and ZNF664 (Lupo et al., 2019). Which doesn't support the causing gene being on chromosome 5 but on 12, but with it not being significant and PITX1 being on chromosome 5 and showing clubfoot symptoms in both humans (when microdeletions occur) and in Pitx1 knockout mice (Alvarado et al., 2011). These between both PITX1 haploinsufficient human and Pitx1 knockout mice similarities in morphology (with the twisted foot), reduced muscle volume as well as reduced artery perfusion can be seen in Figures 5, 6, 7, and 8, in the appendix, all taken from Alvarado et al., 2011. Since PITX1 is a known transcriptional target for TBX4, the T-box transcription factor known to be associated with human clubfoot (Alvarado et al., 2010) it further supports the involvement of PITX1 in human clubfoot. Alvarado et al. does raise an interesting point in their 2011 paper suggesting that the Pitx1⁺⁻ mouse is a better model for human clubfoot than pma mice, as it is missing the common peroneal nerve, while it is present in both Pitx1⁺⁻ mice and humans. This linked with the slight difference in the affected muscles, does raise concerns about the validity of being a human clubfoot model, but there are still too many similarities for it not to be considered as a decent model, in my opinion. Gammy mice, in which the GRIT gene which encodes Rho-GTPase is deleted was another proposed mouse model, but it showed severe brain abnormalities (Sangha et al., 2003) that are not present in human's with CTEV. This example is important for establishing what animal models are probably useful because gammy mice displaying symptoms that are completely absent in human clubfoot makes it a much less accurate tool than that of pma mice who display very similar traits, just not exactly the same. I believe through 'genetic tweaking' of the many genes that are involved in bone, muscle, and nerve development in the foot we can engineer pma mice, or the Pitx1⁺⁻ mice, to have exactly the same traits as human clubfoot, therefore better understanding the genetics/causes of human clubfoot.

Having previously mentioned some neurological defects, this section will examine the neurological hypothesis. Decamethonium Bromide (DB) is a neuromuscular blocking agent that has previously been used to investigate muscle development in paralysed chicken limbs (Macharia et al., 2001; Germiller et

al., 1998), but DB can be used to teratogenically induce idiopathic clubfoot, possibly neurogenically (Kilby and Vargesson, 2009). This DB induced clubfoot showed reduced amniotic fluid volume, absent tendons, reduced muscle volume, and stunted muscle-nerve branches (Kilby and Vargesson, 2009). The nervous system malformations have been observed in genetically modified EphA4 homozygous mutant mice to produce clubfoot like deformities, see figure 9 in the appendix (Helmbacher et al., 2000). When the EphA4 gene is inactivated it causes abnormal navigation during the early stages of limb development, specifically of the lateral motor column (LMC) in the hindlimb and at later stages causes an overall reduction in the amount of motor neurons, seen in human CTEV. However, like the gammy mouse, there have been past experiments that implicate this gene with hindbrain making it likely to cause defect (Nieto et al., 1992; Gilardi-Hebenstreit et al., 1992), calling into question this genetically modified animals' reliability in accurately representing clubfoot, but there was no sign of these defects in the Helmbacher et al. experiment. A 2018 study using pma mice by Collinson et al. has pointed to this growth reduction in this sciatic nerve LMC, involving EphA4, to be the primary development defect. Upon mapping the implicated region, they found overexpression of LIM-domain kinase 1 (Limk1), then using molecular and genetic analysis they found that it acts in the EphA4-Limk1-Cfl1/cofilin-actin pathway, which controls nerve growth cone collapse and extension. This reduction in nerve growth, see figure 10 in the appendix, was then shown to cause the reduced muscle growth, see figure 11, as previously mentioned, and increased apoptosis in the dorsal muscles mentioned previously. This study also ruled out neural tube patterning and showed that it was the reduced extension of motor that causes the neurological defects. They also went further than using pma mice through the electroporation of plasmids expressing LIMK1 into chicken neural tube, which caused the same axon loss as in pma mice, and subsequent clubfoot. I agree with their conclusion that this supports a neuromuscular aetiology for clubfoot, and this indicates Limk1 as one of the genetic components. This therefore means that pma mice are a good model for human clubfoot, and only through more experimentation will we be able to understand the full genetic mechanisms of clubfoot, but it is clearly complex.

We now understand that the EphA4-Limk1-Cfl1/cofilin-actin pathway is involved along with PITX1-TBX4 pathways (which are important in early limb development), but these complex also involve lateral mesoderm HOX signalling (Alvarado 2016). HOXD, HOXC, and HOXA clusters due to their involvement in limb and muscle patterning and has been shown that errors in these can lead to oxidative damage, inflammation and apoptosis (Wang 2018). NAT2 is involved in smoking associated TEV, as it causes less acetylation, which could lead to the build-up of possibly toxic amines and adducts. MYH8, MYH3, TNNI2, TNNT3, and TPM2 are all components of the contractile complex for muscles, also associated with CTEV (Basit and Khoshhal 2017). All of these associated genetic factors and more (including how they were identified), along with the one environmental risk factor of smoking, can be seen in figure 13 in the appendix. These all add to the to the genetic factors above when trying to explain CTEV in humans. A main gene that causes clubfoot has not been found using these methods (genome wide association studies, copy number variations and linkage analysis), and as such, it is my opinion that we must use genetically modified animal models to work towards fully understanding the genetics behind clubfoot.

With this progress of understanding of the genetic mechanisms and causes of clubfoot, a very important question is what we are going to do with this knowledge, it is ultimately to help and treat those who are born with clubfoot. There has been research into developing personalised treatment for patients with clubfoot specifically those resistant to standard treatment by using not only molecular genetic engineering of mouse models of clubfoot but also human gene sequencing with MRI of clubfoot (Dobbs and Gurnett 2017). This means that those with 'treatment resistant clubfoot' can be identified early at or before birth and as such their treatment can be the more extreme initial surgical intervention, instead of starting on the Ponseti method only to have it fail leaving the new-born older and consequently less responsive to treatment (Dobbs and Gurnett 2017). Beyond increasing the initial and subsequent manipulation, as well as surgical interventions, it is important to consider the future preventative methods with pre-implantation genetic screening, or human genetic editing to irradiate clubfoot, as well as the ethical implications. This would mean that blastocysts with the genetics for clubfoot could be selected either to not be implanted, or have their genome edited, and through these two methods eradicate clubfoot. Past societies like ancient nomads and later Spartans viewed 'deformed' children as a burden and would 'lay them out to die' (Strach, 1986). However, within other societies people with clubfoot have been accepted even becoming figureheads from the Greek

god Hephaestus who was depicted with twisted feet (Strach, 1986), and inbred Egyptian pharaohs, including Tutankhamun (Hawass et al., 2010). with the progress of our understanding, and therefore treatment/screening methods, of CTEV it will be up to future societies to determine the fate of those with clubfoot. In conclusion through the research of genetically modified mice and chickens, we have come to develop a genetic understanding of clubfoot in these animals, and we understand that it is extremely complex including many genes across multiple chromosomes. We now understand The PITX1-TBX4 pathway from Pitx1^{+/−} mice and humans involved in CTEV. We also know from pma mice (and chickens) that the EphA4–Limk1–Cfl1/cofilin–actin pathway is involved and points to neuromuscular aetiology. But we understand from that in humans there are many more implicated genes, but through the use of genetically modified organisms we can fully understand the genetics and causes of human clubfoot. Finally, it will be up to future societies to determine the chosen course of action for individuals with clubfoot, whether it be advancement in physical treatment, genome editing or blastocyst selection, genetically modified animals are allowing us to understand the genetics and aetiology needed for clubfoot interventions.

References

- Alvarado, D., Aferol, H., McCall, K., Huang, J., Techy, M., Buchan, J., Cady, J., Gonzales, P., Dobbs, M. and Gurnett, C. (2010). Familial Isolated Clubfoot Is Associated with Recurrent Chromosome 17q23.1q23.2 Microduplications Containing TBX4. *The American Journal of Human Genetics*, 87(1), pp.154-160.
- DOI: <https://doi.org/10.1016/j.ajhg.2010.06.010>
- Alvarado DM, McCall K, Aferol H, Silva MJ, Garbow JR, Spees WM, Patel T, Siegel M, Dobbs MB, Gurnett CA. (2011) Pitx1 haploinsufficiency causes clubfoot in humans and a clubfoot-like phenotype in mice. *Hum Mol Genet*. Oct 15;20(20):3943-52.
- DOI: <https://dx.doi.org/10.1093/hmg/ddq401>
- Alvarado, D., McCall, K., Hecht, J., Dobbs, M. and Gurnett, C. (2016). Deletions of 5HOXC genes are associated with lower extremity malformations, including clubfoot and vertical talus. *Journal of Medical Genetics*, 53(4), pp.250-255.
- DOI: <https://dx.doi.org/10.1136/jmg.2015.103700>
- Basit, S. and Khoshhal, K. (2018). Genetics of clubfoot; recent progress and future perspectives. *European Journal of Medical Genetics*, 61(2), pp.107-113.
- DOI: <https://doi.org/10.1016/j.ejmg.2017.09.006>
- Böhm M. (1929) The embryologic origin of club-foot. *JBJS*; XI:229–259 Collinson JM, Lindström NO, Neves C, Wallace K, Meharg C, Charles RH, Ross ZK, Fraser AM, Mbogo I, Oras K, Nakamoto M, Barker S, Duce S, Miedzybrodzka Z, Vargesson N. (2018) The developmental and genetic basis of 'clubfoot' in the peroneal muscular atrophy mutant mouse. *Development*. Feb 8;145(3):dev160093.
- DOI: <https://dx.doi.org/10.1242/dev.160093>
- Comparini L., Podestà A., Russo C., and Cecchi F. (2019) Effect of inbreeding on the “Club Foot” disorder in Arabian Pureblood horses reared in Italy, *Open Veterinary Journal*, Vol. 9(3): 273–280
- DOI: <http://dx.doi.org/10.4314/ovj.v9i3.14>
- Delgado-Baeza E., I. Santos-Alvarez, A. Martos-Rodríguez (1999) Retinoic acid-induced clubfoot-like deformity: pathoanatomy in rat foetuses. *J Pediatr Orthop B*, 8, pp. 12-18
- Link: <https://www.ncbi.nlm.nih.gov/pubmed/10709591>
- Dobbs, M. and Gurnett, C. (2012). Genetics of clubfoot. *Journal of Pediatric Orthopaedics B*, 21(1), pp.7-9.
- DOI: <https://dx.doi.org/10.1097/BPB.0b013e31825a2a2c>
- Dobbs, M. and Gurnett, C. (2017). The 2017 ABJS Nicolas Andry Award: Advancing Personalized Medicine for Clubfoot Through Translational Research. *Clinical Orthopaedics and Related Research®*, 475(6), pp.1716-1725.
- DOI: <http://dx.doi.org/10.1007/s11999-017-5337-2>
- Duce, S., Madrigal, L., Schmidt, K., Cunningham, C., Liu, G., Barker, S., Tennant, G., Tickle, C., Chudek, S. and Miedzybrodzka, Z. (2010). Micro-magnetic resonance imaging and embryological analysis of wild-type and pma mutant mice with clubfoot. *Journal of Anatomy*, 216(1), pp.108-120.
- DOI: <https://dx.doi.org/10.1111/j.1365-2796.2009.01932.x>
- Farrell, S. A., Summers, A. M., Dallaire, L., Singer, J., Johnson, J. A., Wilson, R. D. (1999). Club foot, an adverse outcome of early amniocentesis: disruption or deformation? CEMAT. Canadian Early and Mid-Trimester Amniocentesis Trial. *Journal of medical genetics*, 36(11), 843–846.
- Link: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1734259/>
- Feldbrin, Z., Gilai, A. N., Ezra, E., Khermosh, O., Kramer, U. and Wientroub, S. (1995). Muscle imbalance in the aetiology of idiopathic club foot. An electromyographic study. *J. Bone Joint Surg. Br.* 77, 596-601.
- Link: <https://www.ncbi.nlm.nih.gov/pubmed/7615605>
- Fritsch, H. and Eggers, R. (1999). Ossification of the Calcaneus in the Normal Fetal Foot and in Clubfoot. *Journal of Pediatric Orthopaedics*, 19(1), pp.22-26.
- Link: <https://www.ncbi.nlm.nih.gov/pubmed/9890281>
- Germiller, J., Lerner, A., Pacifico, R., Loder, R. and Hensinger, R. (1998). Muscle and Tendon Size Relationships in a Paralyzed Chick Embryo Model of Clubfoot. *Journal of Pediatric Orthopaedics*, 18(3), pp.314-318.
- Link: <https://www.ncbi.nlm.nih.gov/pubmed/9600555>
- Gilardi-Hebenstreit, P., Nieto, M. A., Frain, M., Mattei, M. G., Chestier, A., Wilkinson, D. G. and Charnay, P. (1992). An Eph-related receptor protein tyrosine kinase gene segmentally expressed in the

- developing mouse hindbrain. *Oncogene* 7, 2499-506. Link: <https://www.ncbi.nlm.nih.gov/pubmed/8455939>
- Handelman, J. E. and Badalamente, M. A. (1981). Neuromuscular studies in clubfoot. *J. Pediatr. Orthop.* 1, 23-32.
- DOI: <https://doi.org/10.1097/01241398-198101010-00004>
- Hawass Z, Gad YZ, Ismail S, et al. (2010) Ancestry and Pathology in King Tutankhamun's Family. *JAMA*. 2010;303(7):638-647.
- DOI: <https://doi.org/10.1001/jama.2010.121>
- Helmbacher F., Schneider-Maunoury S., Topilko P., Tiret L. and Charnay P. (2000). Targeting of the EphA4 tyrosine kinase receptor affects dorsal/ventral pathfinding of limb motor axons. *Development* 127, 3313-3324.
- Link: <http://www.ncbi.nlm.nih.gov/pubmed/10887087>
- Hoffa A. Lehrbuch der Orthopadischen Chirurgie. Stuttgart: Ferdinand Enke; 1902. Ippolito Ernesto, De Maio F., Mancini F., Bellini D., and Orefice A. (2009) Leg muscle atrophy in idiopathic congenital clubfoot: is it primitive or acquired? *Journal of Children's Orthopaedics*, 3:3, 171-178.
- DOI: <https://doi.org/10.1007/s11832-009-0179-4>
- Ippolito E, Ponseti IV. (1980) Congenital club foot in the human fetus. A histological study. *The Journal of Bone and Joint surgery. American Volume*. Jan;62(1):8-22.
- Link: <https://europepmc.org/article/med/7351421>
- Katoh, H., Watanabe, Y., Ebukuro, M., Muguruma, K., Takabayashi, S. and Shiroishi, T. (2003). Chromosomal Mapping of the Peroneal Muscular Atrophy (pma) Gene in the Mouse. *Experimental Animals*, 52(5), pp.433-436.
- DOI: <https://doi.org/10.1538/expanim.52.433>
- Kawashima, T. and Uhthoff, H. (1990). Development of the Foot in Prenatal Life in Relation to Idiopathic Club Foot. *Journal of Pediatric Orthopaedics*, 10(2), pp.232-237.
- Link: <https://www.ncbi.nlm.nih.gov/pubmed/2312708>
- Kilby E., and Vargesson N. (2009). 06-P034 Determining the developmental basis of idiopathic clubfoot. *Mechanisms of Development*, Volume 126, Supplement, August 2009, Page S13
- DOI: <https://doi.org/10.1016/j.mod.2009.06.260>
- Liu, Z., Li, X., Chen, B., Zheng, C., Zhong, Y., Jia, Y. and Du, S. (2010). Retinoic acid retards fetal and hindlimb skeletal development asymmetrically in a retinoic acid-induced clubfoot model. *Experimental and Toxicologic Pathology*, 62(6), pp.663-670.
- DOI: <https://doi.org/10.1016/j.etp.2010.05.003>
- Lupo, P., Mitchell, L. and Jenkins, M. (2019). Genome-wide association studies of structural birth defects: A review and commentary. *Birth Defects Research*, 111(18), pp.1329-1342.
- DOI: <https://doi.org/10.1002/bdr2.1606>
- Macharia, R., McKinnell, I., Christ, B., Patel, K. and Otto, W. (2004). Decamethonium bromide-mediated inhibition of embryonic muscle development. *Anatomy and Embryology*, 208(1), pp.75-85.
- DOI: <https://doi.org/10.1007/s00429-003-0362-1>
- Merrill, L. J., Gurnett, C. A., Siegel, M., Sonavane, S., Dobbs, M. B. (2011). Vascular abnormalities correlate with decreased soft tissue volumes in idiopathic clubfoot. *Clinical orthopaedics and related research*, 469(5), 1442-1449. doi:10.1007/s11999-010-1657-1
- DOI: <https://dx.doi.org/10.1007>
- Miedzybrodzka, Z. (2003). Congenital talipes equinovarus (clubfoot): a disorder of the foot but not the hand. *Journal of Anatomy*, 202(1), pp.37-42.
- DOI: <https://dx.doi.org/10.1046>
- Nadeem, R., Brown, J., Lawson, G. and Macnicol, M. (2007). Somatosensory evoked potentials as a means of assessing neurological abnormality in congenital talipes equinovarus. *Developmental Medicine Child Neurology*, 42(8), pp.525-530.
- DOI: <https://doi.org/10.1111/j.1469-8749.2000.tb00708.x>
- Nieto, M. A., Gilardi-Hebenstreit, P., Charnay, P. and Wilkinson, D. G. (1992). A receptor protein tyrosine kinase implicated in the segmental patterning of the hindbrain and mesoderm. *Development* 116, 1137-50
- Nonaka, I., Kikuchi, A., Suzuki, T. and Esaki, K. (1986), Hereditary peroneal muscular atrophy in the mouse: an experimental mode for congenital contractures (arthrogryposis). *Exp. Neurol.* 91, 571-579.
- DOI: [https://doi.org/10.1016/0014-4886\(86\)90053-1](https://doi.org/10.1016/0014-4886(86)90053-1)

- Ponseti, I. (1997). Common errors in the treatment of congenital clubfoot. International Orthopaedics, 21(2), pp.137-141.
DOI: <https://dx.doi.org/10.1007>
- Ramachandran, M., Aronson, J. K. (2006). The diagnosis of art: diastrophic dysplasia and Hephaestos. Journal of the Royal Society of Medicine, 99(11), 584–585.
DOI: <https://dx.doi.org/10.1258>
- Sangha HK, Robson JC, Bowen S, et al. (2003) Deletion studies in the gammy mouse. J Pathol 201, 55A.
- Strach E.H. (1986) Club-foot Through the Centuries. In: Rickham P.P. (eds) Historical Aspects of Pediatric Surgery. Progress in Pediatric Surgery, vol 20. Springer, Berlin, Heidelberg.
DOI: https://doi.org/10.1007/978-3-642-70825-1_6
- Yoshimura, N., Fukuhara, N. and Noguchi, T. (1988). Sensori-motor neuropathy associated with congenital bilateral club feet: histological and ultrastructural study of the sural nerve. No To Shinkei 40, 857-861.
Link: <https://www.ncbi.nlm.nih.gov/pubmed/3190934>
- Wang, Y. (2018). Relationship between HOX gene and pediatric congenital clubfoot. Experimental and Therapeutic Medicine. 2018 Jun; 15(6): 4861–4865.
DOI: <https://dx.doi.org/10.3892>
- Wynne-Davies, R. (1964). FAMILY STUDIES AND THE CAUSE OF CONGENITAL CLUB FOOT. The Journal of Bone and Joint Surgery. British volume, 46-B(3), pp.445-463.
Link: <https://www.ncbi.nlm.nih.gov/pubmed/14216453>

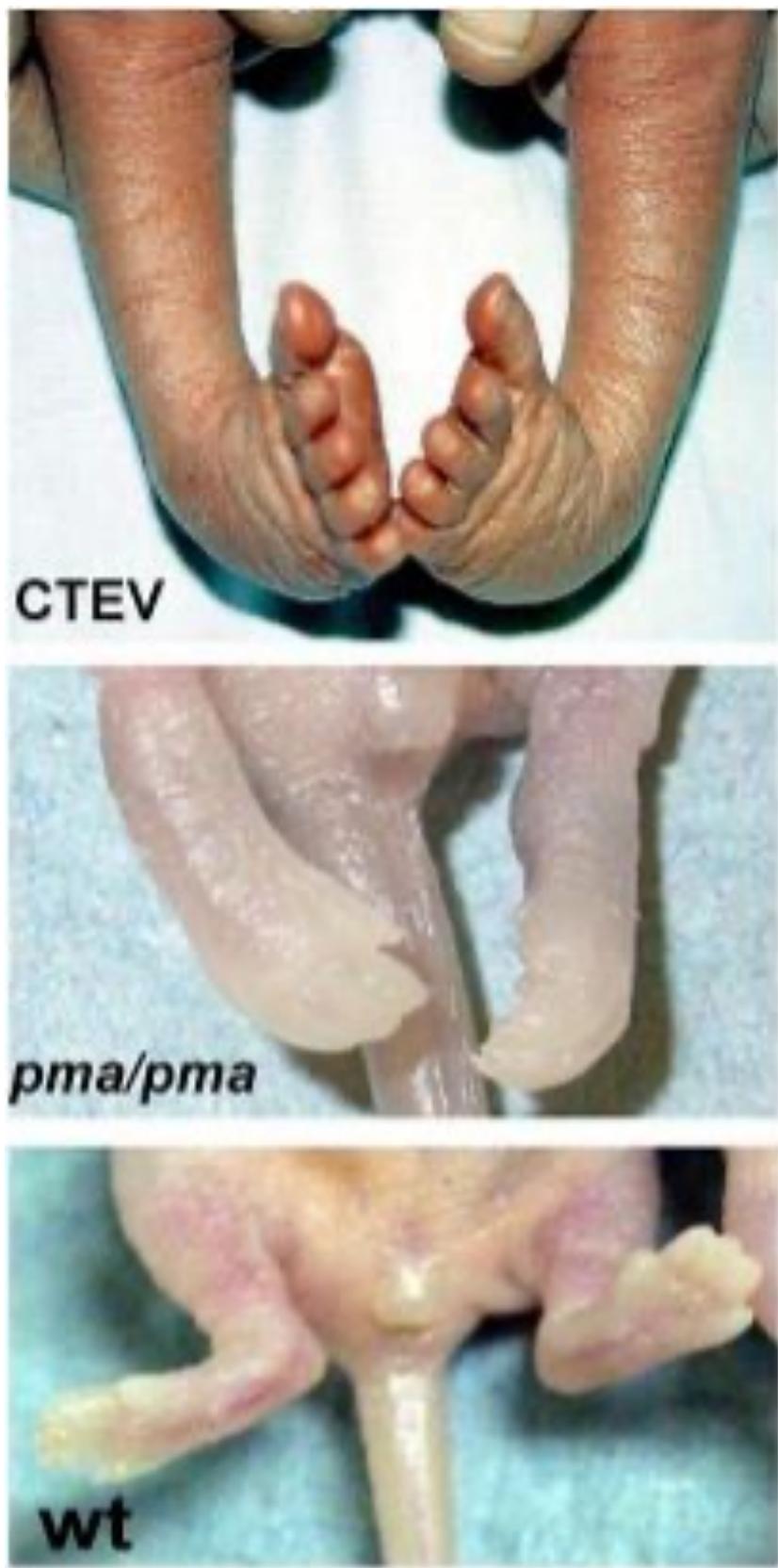


Figure 1: *Clubfoot phenotype in pma/pma mice*. Human newborn with congenital talipes equinovarus (CTEV, commonly known as clubfoot) (top) compared with newborn pma/pma mouse (middle) and newborn wild-type mouse (bottom). From Collinson et al., 2018

Anatomical feature	<i>pma</i> mouse hind limb anatomy	Human CTEV leg anatomy
Tibia and fibula	<i>pma</i> bones same size and shape as wild-type. Distal end of the <i>pma</i> bones not distorted	Not affected
Shank muscle	Reduced	Overall shank muscle reduced
Anterior lateral shank muscles	Hypoplastic in <i>pma</i>	Relatively unaffected
Anterior medial shank muscles	Similar total muscle volume	Relatively unaffected
Posterior shank muscles	Similar total muscle volume	Most affected
Orientation of the foot in sagittal plane	Plantar flexion at the ankle in <i>pma</i>	Equinus deviation
Orientation of the foot in the frontal plane	<i>pma</i> foot shows inversion and adduction of the mid foot and forefoot	Inversion and adduction
Flexor digitorum longus tendons	Appear shorter in <i>pma</i>	May be shorter than normal
Calcaneus, talus, centrale	<i>pma</i> bones same size and shape as wild-type, slight inversion in <i>pma</i>	Variable evidence for changed shape/size
Tarsals	Supination in the <i>pma</i>	Supination
Metatarsal	<i>pma</i> bones are the same size and shape as wild-type, supination with clear adduction in the <i>pma</i>	Adductus
Phalanges	Curled and inverted in <i>pma</i>	May demonstrate latent curling during correction due to shortened flexors

Figure 2: Anatomical characteristics of the hind limb in the *pma* mouse mutants and in human CTEV. From Duce et al., 2010

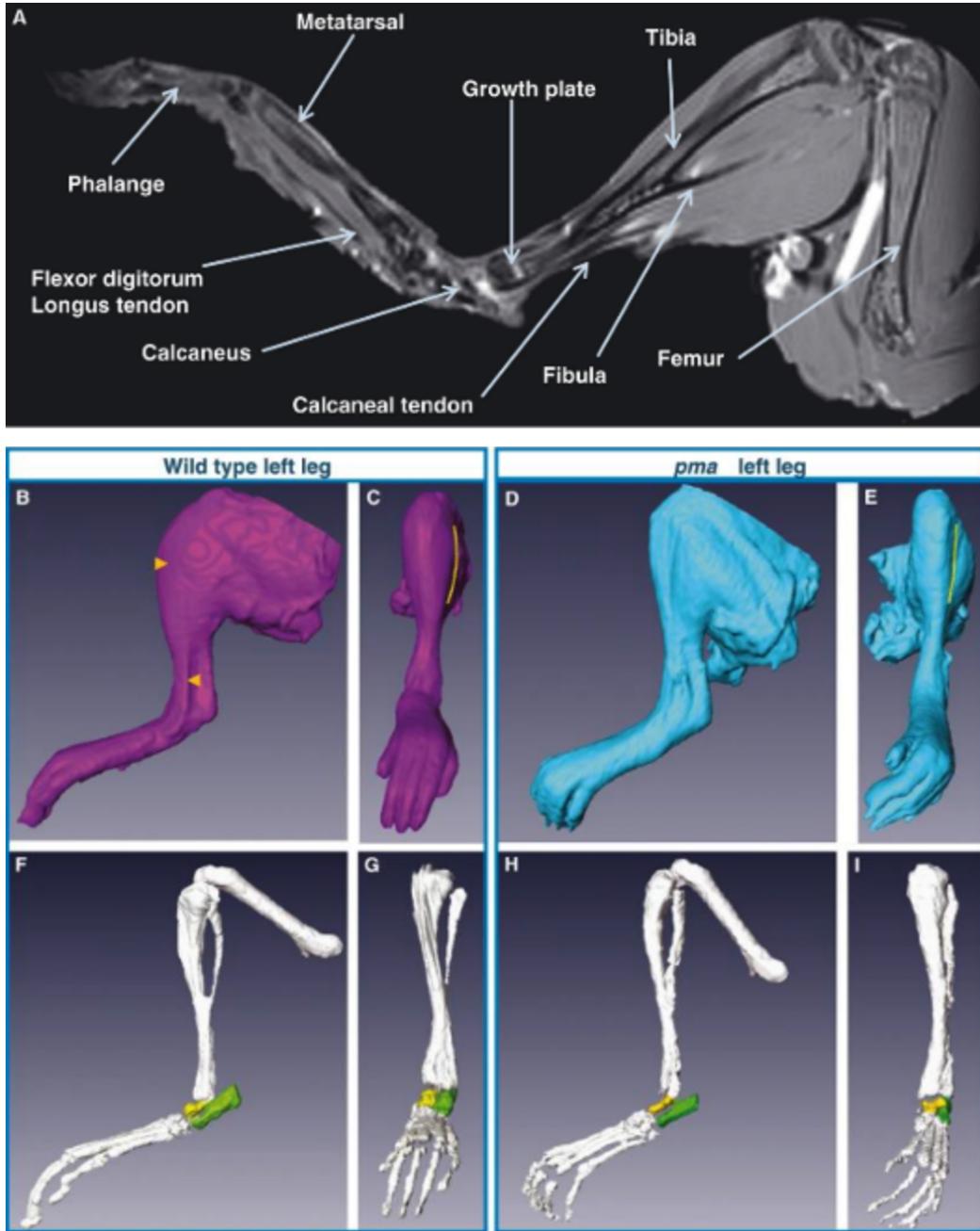


Figure 3: MRI spin-echo images of left legs of 3-week-old wild-type and pma mice; five pairs of limbs were studied. (A) 2D slice in sagittal plane from a TR/TE = 1000/40 ms spin-echo 3D MRI data set of wild-type leg showing main anatomical features. (B,C) 3D surface reconstruction of outer surface of wild-type leg from TR/TE = 1000/6 ms spin-echo image data set. (D,E) 3D surface reconstruction of outer surface of pma mouse leg from TR/TE = 1000/6 ms spin-echo image. (F,G) 3D surface reconstruction of mineralized bones of wild-type leg from TR/TE = 1000/40 ms and TR/TE = 1000/6 ms spin-echo image data sets. (H,I) 3D surface reconstruction of mineralized bones of pma mouse leg from TR/TE = 1000/40 ms and TR/TE = 1000/6 ms spin-echo image data sets (matrix size, $256 \times 256 \times 256$; field of view, $25 \times 25 \times 25$ mm; voxel dimensions, $97 \times 97 \times 97$ m). (B,D,F,H) Lateral views of sagittal plane; (C,E,G,I) anterior views of frontal plane. Dashed line in C and E outlines anterior lateral side of leg (i.e. shank); note reduction in mutant. Calcaneus shown in green and talus in yellow in F,G,H and I. From Duce et al., 2010

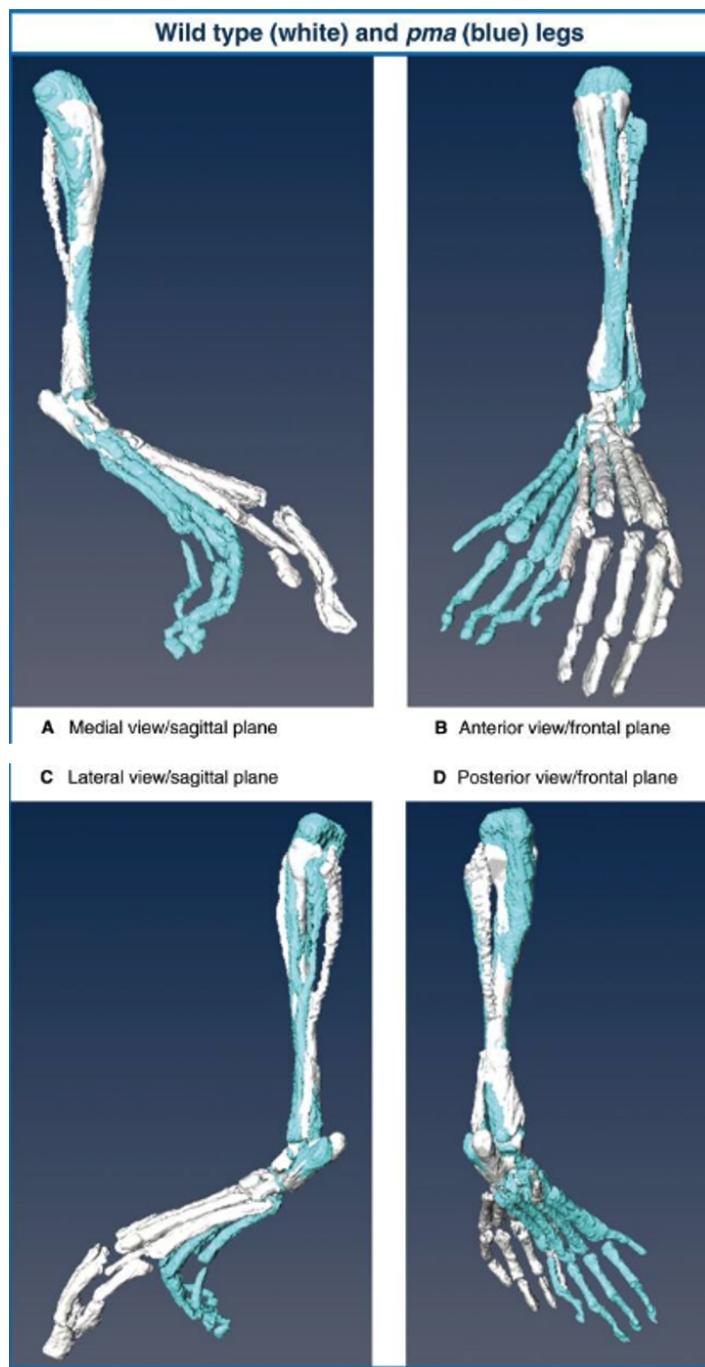


Figure 4: 3D surface reconstructions of mineralized bones from MRI of left legs of 3-week-old mice [wild-type (white) and *pma* (blue)]. Images aligned relative to the tibial mechanical axis to allow direct comparison of anatomy. Three *pma* and wild-type hind limbs were acquired and overlaid; all comparisons showed the same trends. (A) Medial view of sagittal plane, (B) anterior view of frontal plane, (C) lateral view of sagittal plane and (D) posterior view of frontal plane. Mineralized bones of wild-type and *pma* legs reconstructed from TR/TE = 500/6 ms and TR/TE = 500/40 ms spin-echo image data sets and a TR/TE = 250/2.3 ms gradient-echo image data set (matrix size, 256 × 256 × 256; field of view, 25 × 25 × 25 mm; voxel dimensions, 97 × 97 × 97 m). From Duce et al., 2010

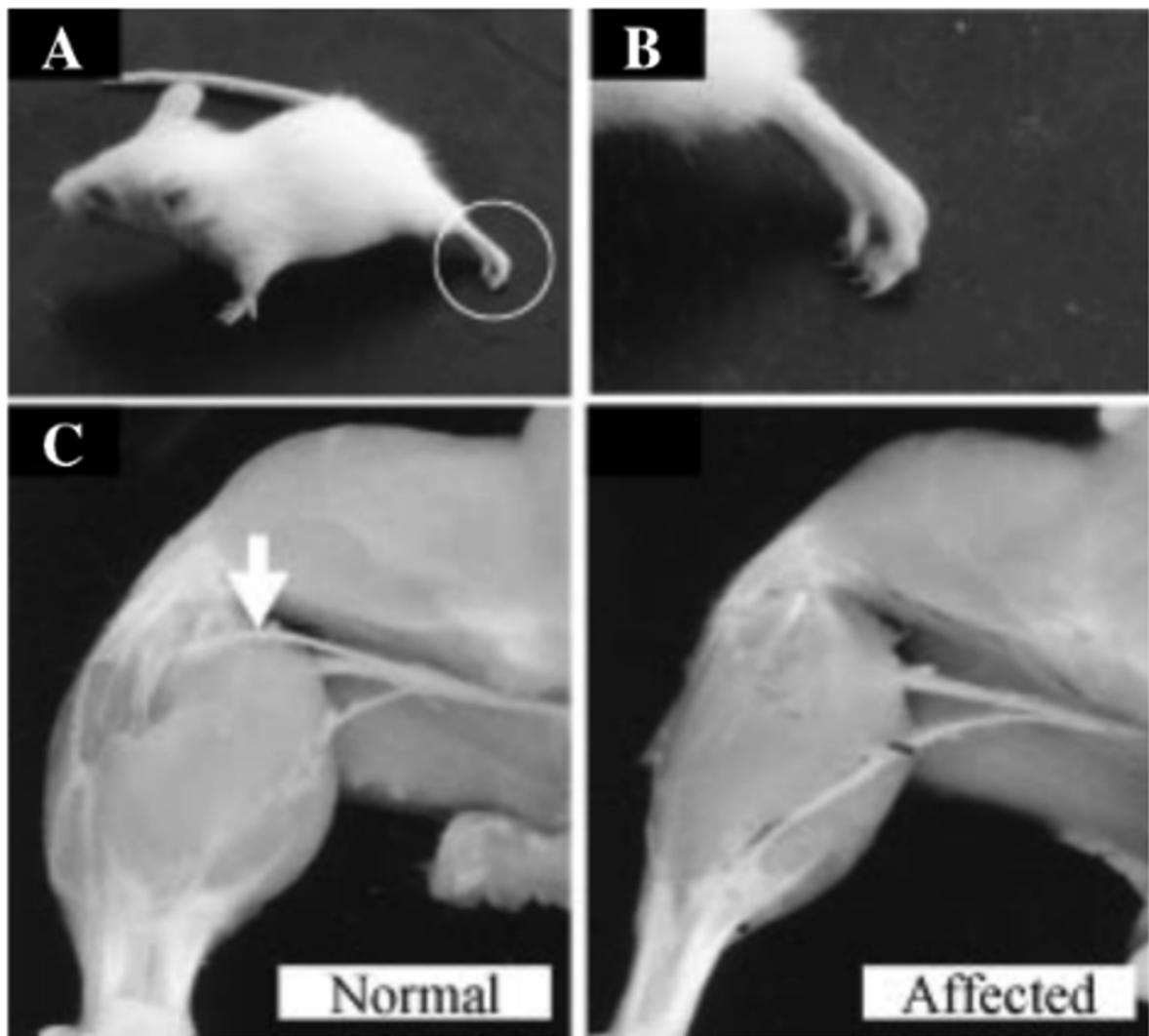


Figure 5: Morphological characteristics of the PMA (peroneal muscular atrophy) mouse. A: A PMA mouse with peroneal muscular atrophy in hind limbs, B: Clubfoot caused by absence of the common peroneal nerve, C: Developed common peroneal nerve (shown by white arrow) in the normal mouse, and D: Underdeveloped common Peroneal nerve in the abnormal mouse. From Katoh et al., 2003

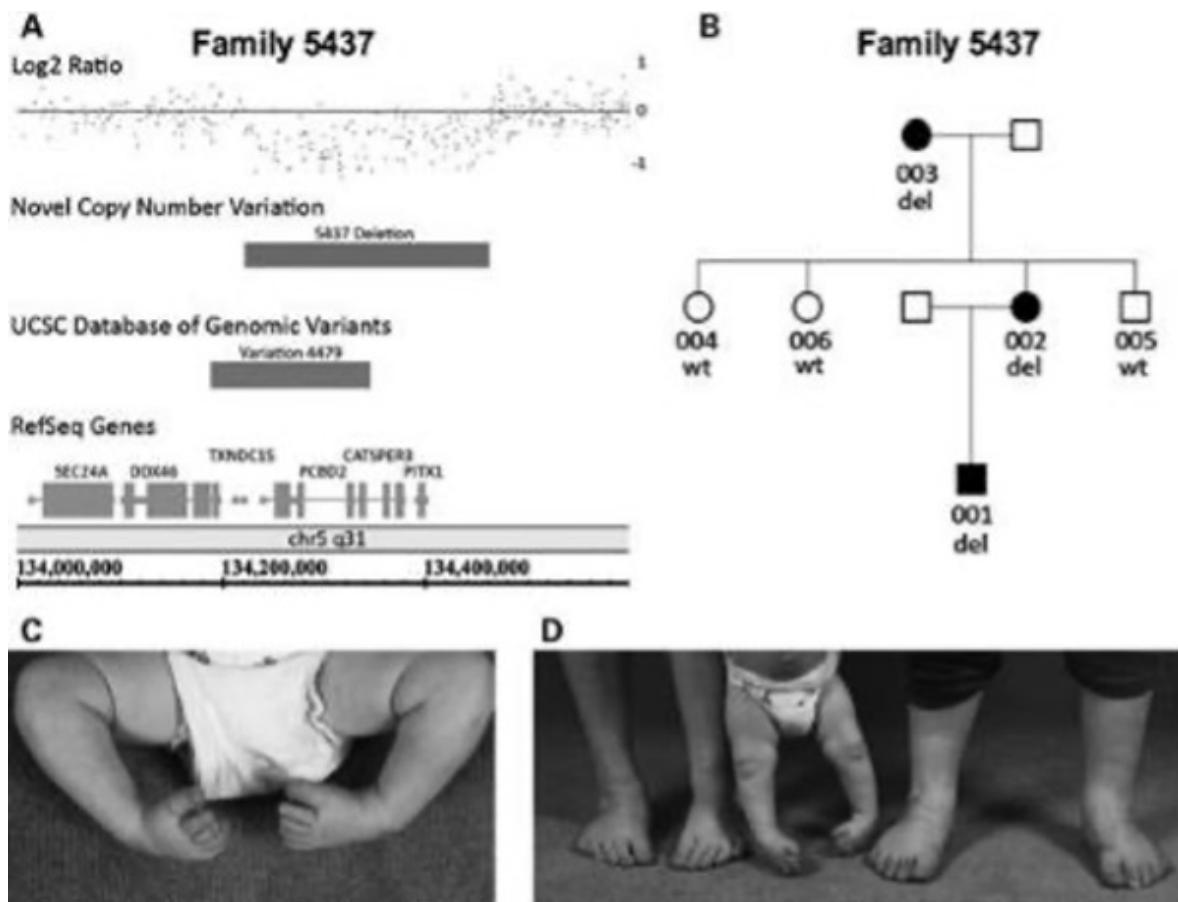


Figure 6: Chromosome 5q31 microdeletion containing PITX1 is present in family with isolated clubfoot. (A) Chromosome 5q31 region showing 241 kb deletion at chr5:134222383–134463022 (hg18 build of the UCSC genome browser), involving 124 markers with decreased log₂ ratios that was detected in the proband. Four RefSeq genes are located within the interval, including PITX1. (B) The chromosome 5q31 microdeletion segregates with clubfoot in family 5437. Black affection status indicates isolated clubfoot, del indicates deletion and WT indicates normal copy number. (C) Proband from family 5437 with untreated bilateral clubfoot. (D) Three generations of family 5437 with the chromosome 5q31 microdeletion showing surgically treated clubfoot in mother (left), untreated proband (middle) and surgically treated maternal grandmother (right). From Alvarado et al., 2011

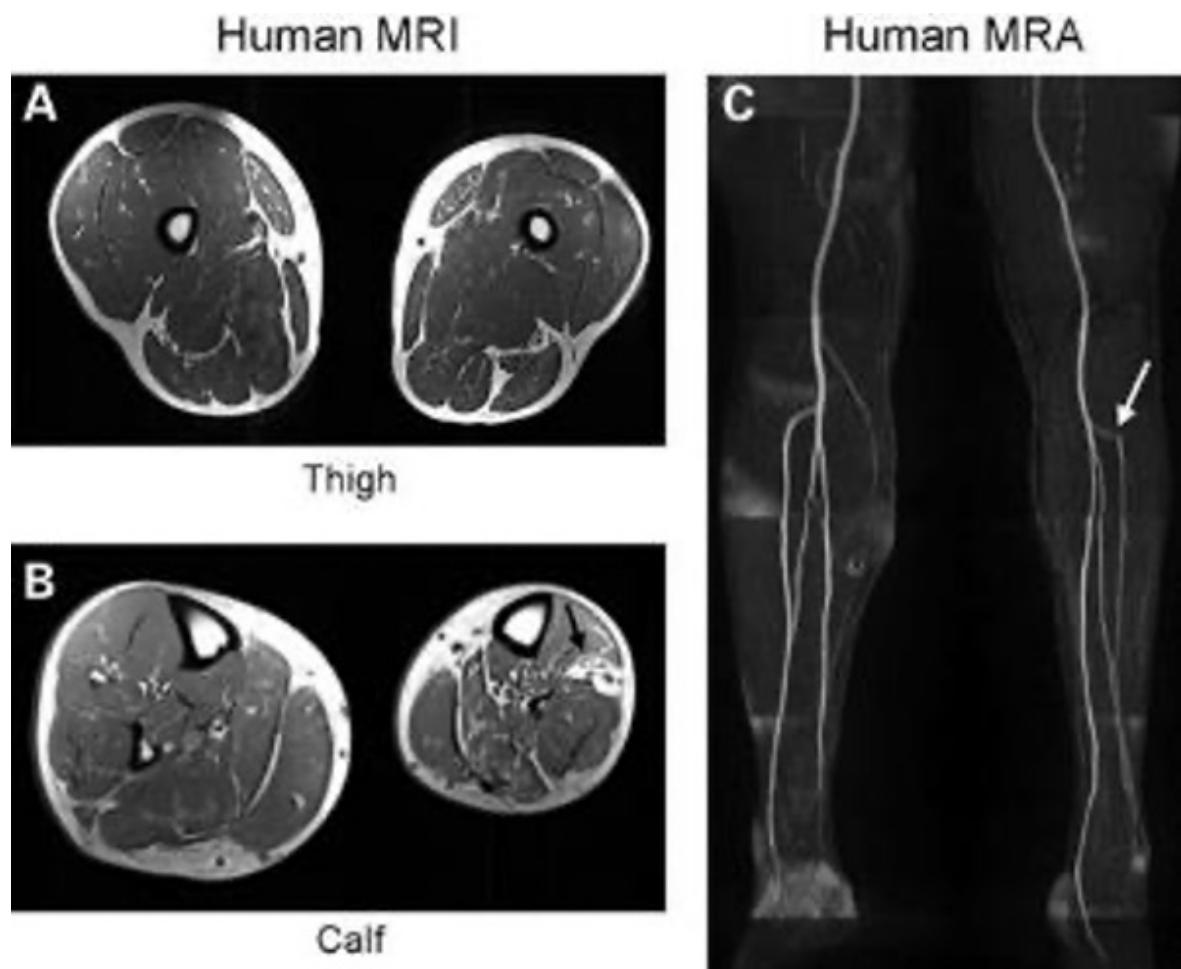


Figure 7: Muscle volume and vascular abnormalities in the affected clubfoot limb of an adult patient with PITX1 E130K mutation. (A) MRI shows hypoplasia of the left clubfoot limb (shown on the right) compared with the unaffected right leg on transverse sections. (B) Images obtained at calf level show more severe involvement than those taken at the thigh. Muscle hypoplasia is present throughout but is most prominent in the lateral and anterior compartments that also show a corresponding increase in fat tissue (black arrow). (C) Magnetic resonance angiograph of same patient showing reduced perfusion of the anterior tibial (white arrow) and peroneal arteries on the affected left leg compared with the right. From Alvarado et al., 2011

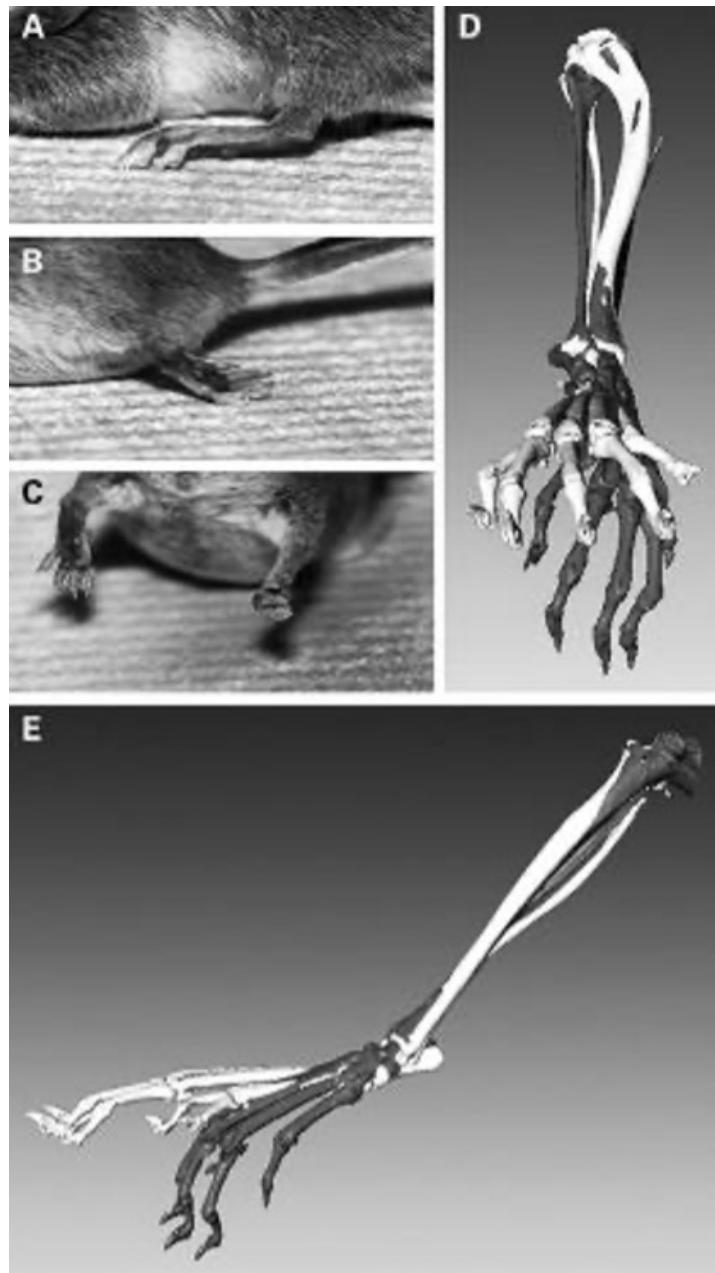


Figure 8: Clubfoot-like phenotype is present in some Pitx1 haploinsufficient mice. (A) Lateral view of unaffected hindlimb of Pitx1^{+/+} mouse. (B) Lateral view of affected hindlimb of Pitx1^{+/+} mouse. (C) Dorsal view of unaffected (left) and clubfoot-affected (right) hindlimbs. (D) MicroCT image comparing clubfoot-like right limb of an affected mouse (gray) to unaffected right limb of a control mouse (white) demonstrating forefoot cavus in the frontal plane and (E) hindfoot equinus in the sagittal plane. From Alvarado et al., 2011

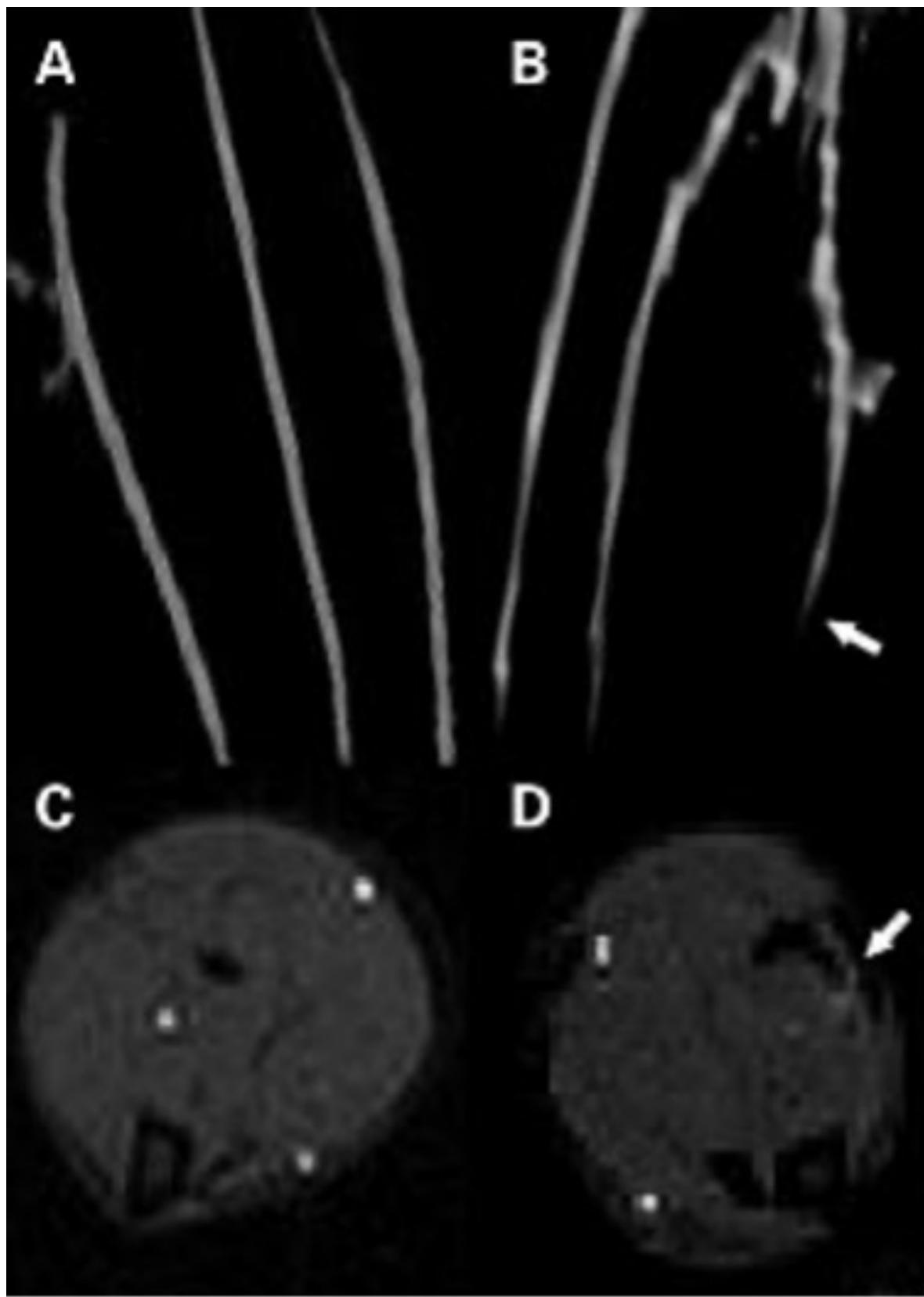


Figure 9: Peroneal artery hypoplasia is present in the clubfoot-like limb of *Pitx1*^{+/+} affected mice. Magnetic resonance angiograph of unaffected (A and C) and affected (B and D) *Pitx1*^{+/+} hindlimbs. The peroneal artery (white arrows) is hypoplastic in affected hindlimbs. There are no apparent anomalies in the anterior tibial or posterior tibial arteries. From Alvarado et al., 2011



Figure 10: (F) EphA4^{+/−} P5 mouse showing an abnormal positioning of the left hindlimb (arrow). From Helmbacher 2000

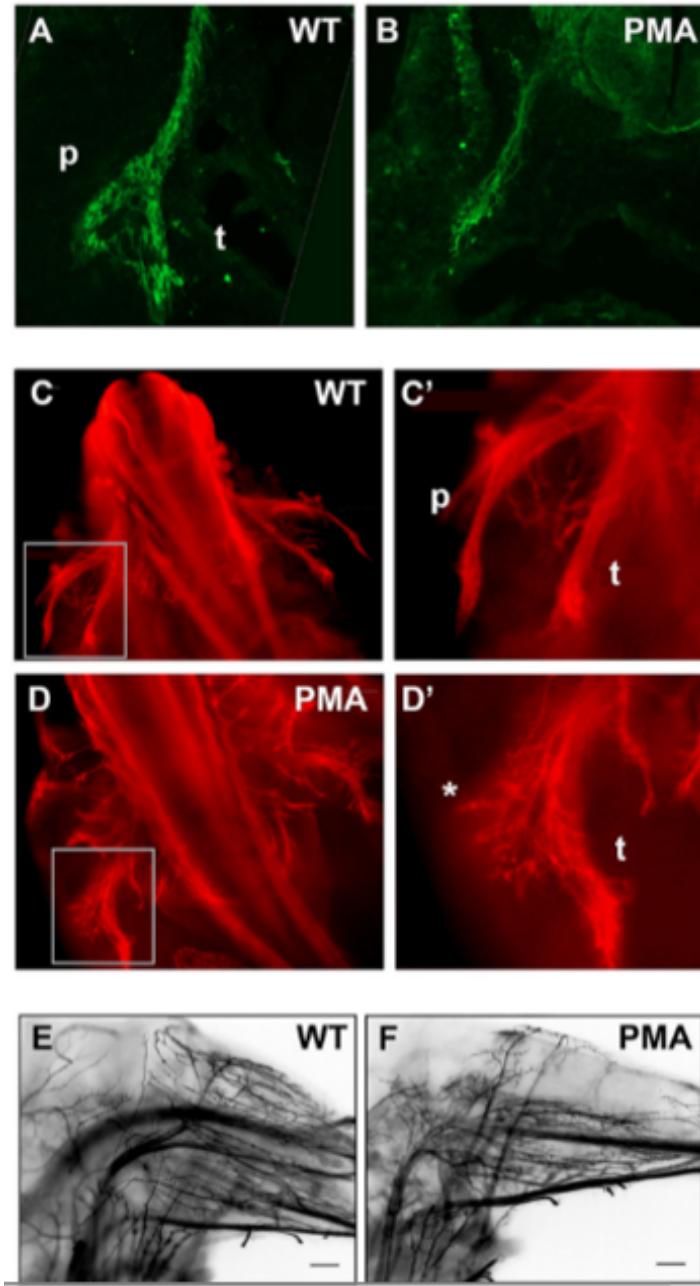


Figure 11: Retardation of nerve growth and abortive innervation of dorsal muscles in PMA mice. (A,B) -III-tubulin immunohistochemistry (green) in transverse sections of stage-matched E11.75 wild-type (WT; A) and pma/pma mice(B). The wild-type sciatic nerve has projected further than that in the pma/pma mice and, unlike the PMA nerve, started to branch into discrete dorsal (peroneal, p) and ventral (tibial/sural, t) components. (C,D) Whole-mount -III-tubulin immunohistochemistry (red) on wild-type (C,C) and pma/pma (D,D) embryos. C and D show magnifications of the boxed areas over the left-hand side nerves in C and D, respectively. The peroneal (p) and tibial/sural (t) components are labelled. In pma/pma embryos, the tibial/sural branch is grossly normal, but only a few defasciculated axons are observable (asterisk) in place of the peroneal nerve, presenting a feather-like appearance. (E,F) Whole-mount -III-tubulin immunohistochemistry on lower hindlimbs of E16.5 wild-type (left) and pma/pma embryos (right). Dorsal is to top: the dorsal muscles of the pma/pma foetuses are completely aneural, suggesting that the putative peroneal axons noted at E12.5 have not survived. Scale bars: 50 μ m. From Collinson et al., 2018

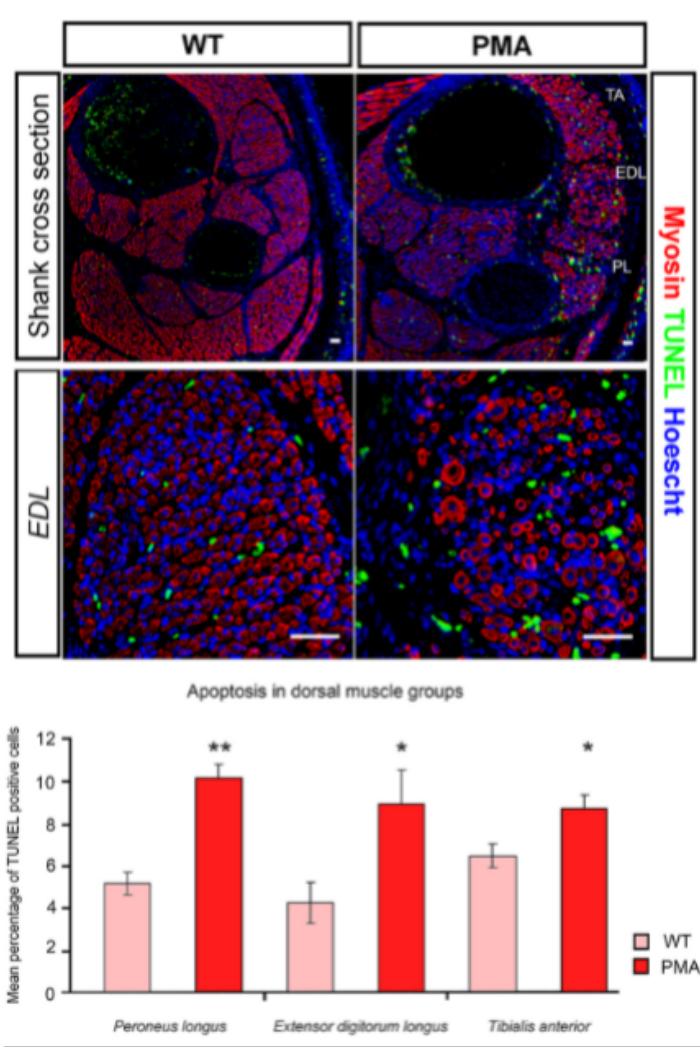


Figure 12: Increased apoptosis in dorsal muscleblocks of the pma hindlimb. Top: TUNEL labelling (green) to visualise apoptotic cells in cross-sections of E16.5 wild-type (WT; left) and pma/pma foetuses (right), combined with immunohistochemistry for myosin heavy chain (red) and Hoechst nuclear stain (blue). Higher magnification of the one dorsal muscle, the extensor digitorum longus, is shown. Bottom: Although apoptosis occurs in all muscles, the percentage of TUNEL-positive cells was significantly greater in the three major dorsal muscle blocks of pma/pma foetuses than in wild-type controls ($n=8$ for both groups). * $P<0.05$; ** $P<0.01$. Error bars represent s.e.m. Scale bars: 50 μ m. From Collinson et al., 2018

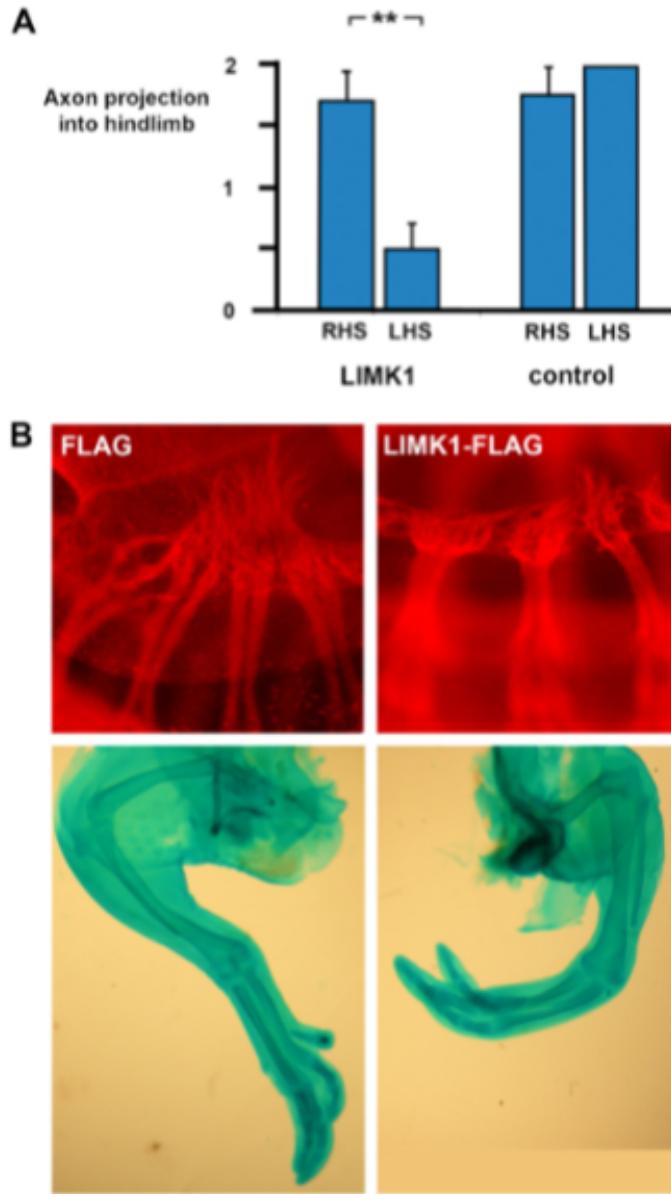


Figure 13: Electroporation of LIMK1 into chicken neural tube causes axon loss and clubfoot. Electroporation of plasmids expressing LIMK1 or empty vector controls into HH stage 11 chickens followed by immunohistochemistry for -III-tubulin 72 h later or Alcian Blue cartilage staining after further 5 days. (A) Nerve projection scored 0-2 as described in the Materials and Methods for electroporated (left-hand side, LHS) and non-electroporated contralateral sides (right-hand side, RHS) of each embryo, for LIMK1 and control vectors, and shows significant inhibition of axon growth in LIMK1-treated nerves, but not in empty-vector electroporations. LIMK1 electroporation: RHS nerve score=1.67±0.25, n=9; LHS score=0.5±0.22, n=8 (one embryo damaged cf. RHS). Control FLAG electroporation: RHS nerve score=1.75±0.25, n=4; LHS score=2±0.00, n=3. **P=0.001 (paired t-test). Error bars represent s.e.m. (B) (Top) Whole-mount -III-tubulin immunohistochemistry (red) on chicken embryos showing normal sciatic plexus formation and axon projection (score 2) after an empty ‘FLAG’ vector transfection compared with representative failure of nerve plexus formation (score 0) after LIMK1 transfection. (Bottom) Alcian Blue cartilage preparation of (left) a control FLAG-electroporated chicken limb and (right) limb of one of the chickens (3/12) that exhibited a mild clubfoot-like phenotype after transfection with LIMK1. From Collinson et al., 2018

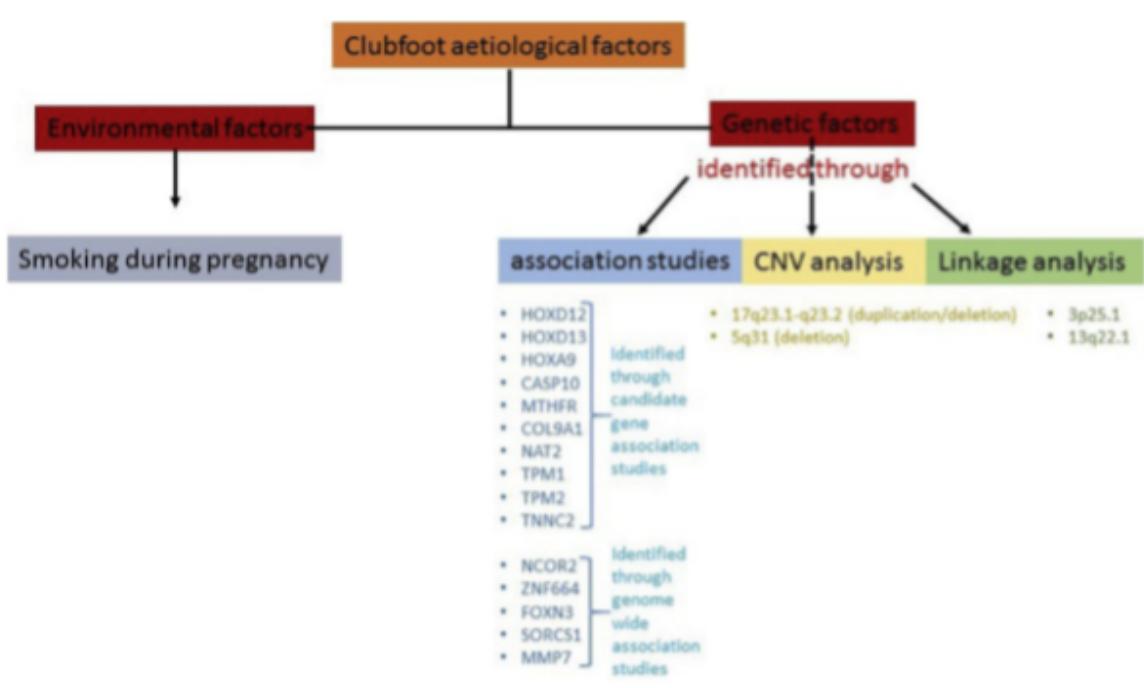


Figure 14: Overview of environmental and genetic factor associated with [human] clubfoot. From Basit and Khoshhal 2017

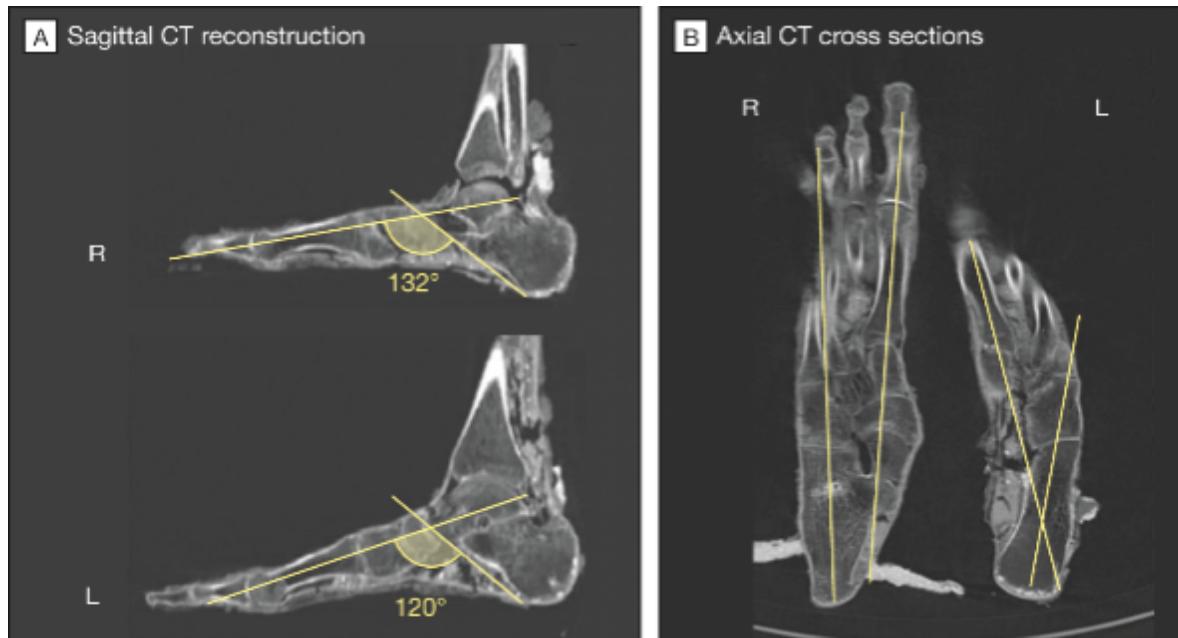


Figure 15: Analysis of malformations in the feet of Tutankhamun A, As indicated by the angle between the axis of the first metatarsal and the line between the lowest point of the calcaneal tuberosity to the lowest point of the calcaneocuboid articulation (Rocher angle), the arch of the right foot is flat (132°) compared with that of the left (120°). The Rocher angle of a normal foot is 126°. B, The supine and inwardly rotated position of the left foot are further features of clubfoot. From Hawass et al., 2010

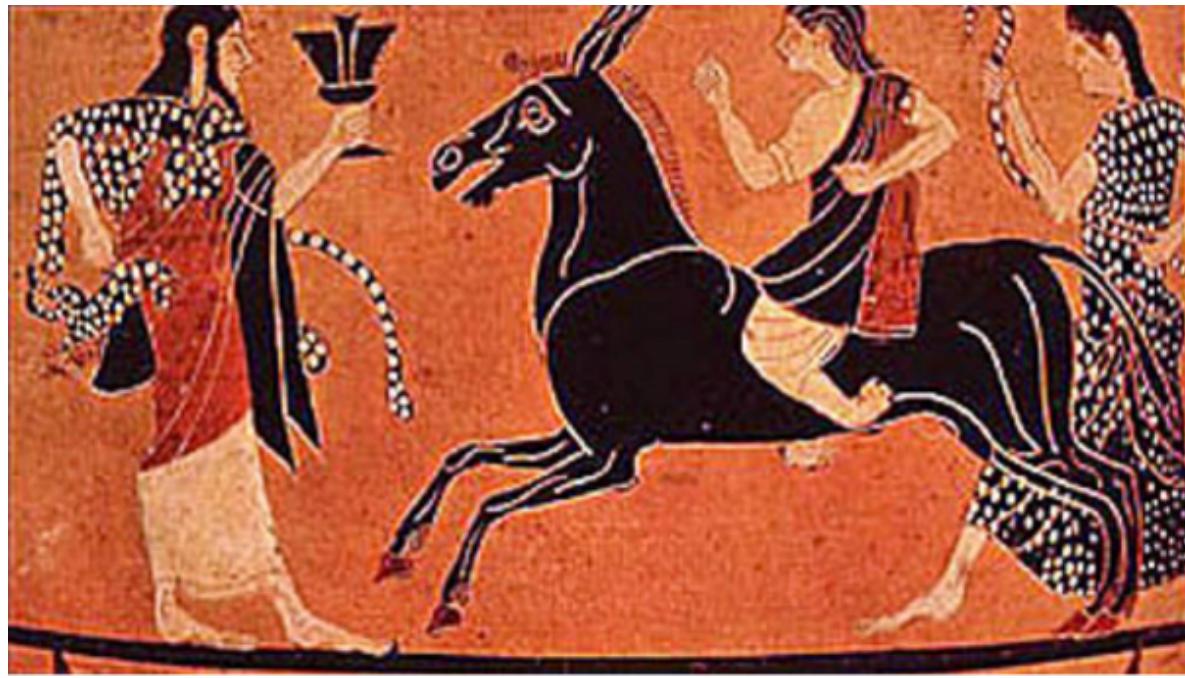


Figure 16: Hephaistos (mounted) with Dionysus (left) as depicted on a sixth century BC hydria (water-vase). (Image reproduced in colour online.). From Ramachandran, M., Aronson, J. K. (2006)

My daughter wants a pet cloned panda for her birthday.
Explain how cloning of mammals is achieved, why people want
to do it, and the circumstances under which it might be
allowed. Discuss why Lizzie should or should not get her panda.

Paul Shepherd

February 9, 2023

Cloning is the production of genetically identical copies of biological material, from genes to entire organisms. These “clones” can occur naturally in asexually reproducing organisms such as; Planaria (e.g. *Dugesia Japonica*), Hydra (e.g. *Hydra attenuata*), starfish (e.g. *Coscinasterias tenuispina*), lizards (e.g. *Aspidoscelis neomexicanus*) through; binary fission, budding, fragmentation and parthenogenesis respectively. Pandas (giant pandas) cannot naturally produce clones, like all mammals that reproduce sexually, so producing a cloned panda would require artificial cloning. This artificial cloning of mammals has been developing since the 1970s with nuclear-transfer methods involving ovulated rabbit eggs. This developed into successfully cloning sheep/mice/cattle from blastomere nuclei, before the successful cloning using adult cells through somatic cell nuclear transfer like Dolly the sheep. Now independent companies can use this technique to clone pets (dogs, cats etc.) for huge amounts of money (Approx. 50,000-100,00 USD). So, this could also apply for pet giant pandas, which are notoriously bad for reproducing naturally. Researchers have actually been working on cloning pandas to increase the low numbers (2,000), with some experiments in 1999 and plans announced in 2004, which gained publicity again in 2015 when the same group that cloned Dolly took panda tissue samples to work towards cloning. With cloning a pet panda now established as not simply science fiction this essay will explain how to clone mammals, why people want to do it, the circumstances it might be allowed, before discussing why Lizzie (or anyone) should or should not have a cloned pet panda.

Mammal cloning through somatic cell nuclear transfer into an enucleated oocyte is achieved in 6 steps. First (1) take a somatic cell from the animal you want to be cloned that contains its complete genome, this could be anything from an embryonic stem cell to a skin cell to a mammary cell (which was the case for Dolly). Then (2) make an enucleated oocyte by taking the pronucleus out of an unfertilised oocyte (of the same species, although it has been done experimentally with different species). Then (3) take the nucleus out of the donor somatic cell and put it into the enucleated cell either by electrofusion or direct injection, which allows the donor DNA to act as the two meeting pronuclei in normal sexual fertilisation. The oocyte then needs to be (4) ‘activated’ by chemical or electrical activation, which causes the oocyte to start acting as if it has been fertilised starting the calcium transients. But activation is not quite equal to natural fertilisation an example of this is the fact that downregulation of the inositol-1,4,5-triphosphate receptor did not occur during activation while it does during normal fertilisation. The cytoplasm of the host cell also decondenses the DNA, causing a loss of silencing through things like heterochromatin, allowing it to become totipotent again (meaning it changes from the possibly specialised function to a form where it can make all the cells of the future organism again). Then just as in vitro fertilisation (IVF), some of the cells survive, divide and start to develop as normally fertilised oocytes, which like IVF are selected for and (5) implanted into the uterus of a pseudo-pregnant female (again of the same species). Where the developing clone (6) completes development as normal in utero. Dolly in 1996 was proof that this worked (even if it was 1/100), but to give a panda related example Chen took a somatic cell from a giant panda, transferred the nucleus into an enucleated rabbit oocyte and later a cat oocyte, and then implanted the cat oocyte into a cat womb. Polly in 1997 was proof of that we can clone transgenic (genetically manipulated) animals, so in the context of pandas we could use modern gene editing tools like CRISPR/Cas9 to change the clones to make them comparatively more fertile and/or libidinous, ending their reproduction problems, but

would probably make many unexpected problems including off target effects and behavioural issues.

People want to clone mammals for a number of reasons, one relating to the previously mentioned transgenic clones is that in commercial agriculture you can produce superior animals designed with specific properties that you want, e.g. cows that produce the perfect wagyu or huge amounts of milk. These could then be further genetically modified to act as bioreactors, that can produce useful macromolecules or proteins that are useful in human medicine, Polly was an example of this as she expressed the human clotting factor IX in her milk, which could then be extracted and intravenously given to people suffering from haemophilia B. This so called “pharming”, although more commonly uses bacteria or yeast as bioreactors (often producing vaccines including hepatitis vaccines and human papillomavirus) there is clear progress in the large-scale use of mammalian bioreactors, with a 2017 example of 200 transgenic cloned cows producing recombinant human lactoferrin without any markers were made. Pigs have been genetically modified to reduce cross species immune barrier to allow for xenotransplantation to meet the organ transplant demand, which is so great that 18 patients in Europe die each day waiting for a transplant. Further adapting pigs to produce perfectly accepted organs or tissues for transfer then cloning them is a serious interest for cloning mammals. This would get around the extreme fears presented in the film “The Island” where human clones are produced for organ harvesting, which is clearly unethical, but also points out that some people want to clone mammals so that we can ultimately work towards cloning ourselves, just as we use animal models to study development, not necessarily because we are interested in a frogs/chickens/mice development (although people might be), but that they are good models for our own development. So mammalian clones could be useful for generating increased amounts of data whether it be with; genes (through PCR), cells (through embryonic stem cells or induced pluripotent stem (iPS) cells, through the forced expression of key transcription factors e.g. c-Myc, Oct4, Sox2 and Klf4), tissues (through tissue engineering), or organisms (through artificial cloning), this would mean that researchers could make what they want and follow its progress without having to wait for things like specific biopsies. Cloning mammals therefore gives us good models to study biology including embryonic development, cancer biology, stem cell biology, evolutionary biology allowing for faster breakthroughs in subjects like aging/immortality. Research in this area has made tailor-made stem cell therapy a realistic possibility, where you can take somatic cells from an ill person, put it into a host oocyte to make a blastocyst stage clone, which you can harvest the inner cell mass to make embryonic stem (ES) cells, which you can then differentiate (using chemicals, cytokines etc.) into whatever tissue you want to then use to repair the individual that you harvested somatic cells from. This concept has been proven possible with Parkinson’s as ES cells have been engineered to make functional dopamine neurons, which have been put into Parkinson’s model rats improving motor coordination therefore treating Parkinson’s. People may also want to clone mammals to resurrect or have multiple of their loved pet, which we know can be done, Barbra Streisand’s dogs or Huang Yu’s dead cat for example. So, it is not unfeasible that some people would want to clone people to make clone army (military, like in Star Wars, or army of friends, like I would like) or clone a dead leader (Mao, Lenin, or other non-communists) or great (Elvis, Newton, Darwin etc.). Since de-extinction was proven possible, if limited with its 7 minutes of life, after cloning the extinct Pyrenean ibex by taking the nucleus of a somatic cell, into a goat oocyte, and implanted into a goat.

Just because something is possible it doesn’t mean it should be done, medical research is inseparable from ethics because of its goal to improve humanity, this section will discuss the circumstances cloning mammals might be allowed. Just as Nazi’s conducted atrocious medical research on concentration camps inmates, and Americans conducted the Tuskegee syphilis study (looking at 400 untreated syphilis patients vs 200 controls), we too could find ourselves harming human or mammalian clones in the name of research, which is why we must not only abide by our own ethics but also The Nuremberg Code, the ethical boards of the research institutes and country (as well as its laws). I think every researcher would/should hate to produce a piece of research that is a moral enigma such as Pernkopf’s atlas (an anatomy book made by Nazi doctors), as it was produced under horrible circumstances but could be having a net good, but whether you look at it utilitarianly or through categorical imperatives it is up to you. As I am an undergraduate (medical sciences not philosophy) student in an exam I will base the circumstances on when mammalian cloning is allowed on the experiments that are published after being approved by ethics boards that I can remember and write down. Human cloning and stem cell research are huge and heated ethical debates as it is potentially destroying/creating life and since the discovery of iPS cells making all cells have the potential to create life, it makes this debate either more

complicated or has got around it, as the scientific community allows extensive iPS cell research in the pursuit of therapies I would say its got around it. Animal cloning and genetic manipulation is allowed as shown by the above examples, as these experiments (and others like it) are arguably having a net good and respect the 3Rs of animal research (Replace, Reduce, Refine) as well as the possible 4th R (Responsibility to promote animal welfare, as we are taught it as undergraduates and animal ethical approval is on every paper involving animals). I am unsure if it ethically ok to de-extinct an animal like the Pyrenean ibex only to have it die of lung failure 7 minutes later, that might be unnecessary suffering, just as resurrecting dead pets or cloning new pets might create unnecessary suffering making ethical problems, also they do not have ethical approval, and just because there are no laws shouldn't make it fine. On topic of unnecessary suffering almost all clones die sooner than their non-cloned counter parts as cloning is not without its problems these include; imprinting (like methylation inconsistencies on genes of the somatic cell interfere with expression issues), reprogramming (of the donor nucleus must be reprogrammed by the oocytes cytoplasm, optimum might be 50 percent of genes being expressed or silenced, if this process is incomplete it results in premature death), telomeres (being shorted in the "aged" somatic cells, means that they have a reduced life span), and mitochondria (as clones have mitochondrial heteroplasmy this can create issues). With further perfection of these cloning limitations it makes all forms of mammalian cloning more ethical but does not address main ethical issues of animal research and playing God, and ownership with human cloning.

Finally, why Lizzie should or should not get her pet panda is up to you as a parent (and the better scientist), but here are some arguments. Pet cloned pandas should be a thing because; working towards cloning pandas has received ethical approval for over two decades, cloning pets is actively practiced (legally), there are only 2,000 panda remaining with reproduction (naturally/assisted) being problematic so maybe introducing cloned pandas as pets can increase the global population like big cats where some species have more alive as pets as in the wild. Pet cloned pandas should not be a thing because; pandas are not pets they are all owned by the Chinese Government and used as political currency so I'd prefer to avoid that headache of ownership that could get as bad as human clones, the numbers of pandas are increasing and they are no longer endangered just vulnerable, also it does not address cause of their reduction which is the reduction of habitat, the ridiculous costs of cloning not just pandas as pets but all pets could be spent on better things like; research with an actual purpose (cloning mammalian bioreactors, stem cell therapies, anything trying to cure diseases), medical aid (vaccines), or just getting food to areas with man-made famines.

In conclusion, whether Lizzie should get her panda is up to you, it is possible to clone mammals through the above steps, and there are many reasons to clone mammals, but it is an ethical minefield with cloning humans, pharm/farm animals, and pets each having their own concerns. The technology of cloning and genetic manipulation is not perfect resulting in early death and possible unnecessary suffering but is improving to the point where pet pandas might be possible by the time I have (grand)children but if they want a pet panda they will have to pay for it and deal with it themselves after they have moved out.

Describe some evolutionarily important mutations in developmental genes that have occurred during human divergence from other apes. State how they are thought to have affected our phenotype and use examples of changes in both coding and regulatory regions of genes.

Paul Shepherd

February 9, 2023

Humans are just another branch of great apes, we are genetically closest to chimpanzee as somewhere between 5 and 6 million years ago we shared a common ancestor which does not leave a large amount of time for genetic change. This means that the fact that 96 percent of human and chimp being directly comparable does not come as a massive surprise, but there are at least 35 million single base pair changes 5 million larger base pair changes. Some of these changes are in developmental genes that have occurred during human divergence, which is the first phase of becoming different from chimpanzees, the second phase is the changes associated with colonisation out of Africa and the 3rd phase is recent evolution since farming. This essay will focus on this first phase, specifically focussing on big brains, speech, weaker bodies and immune systems.

We have many regulatory regions changes, human evolution has been rapid, bringing us a bigger brain and a weaker body, compared to chimps. The things that seem to be the main difference are not the coding sequences but the changes in the regions responsible for regulation. This could be justified by the areas of genomes that are highly conserved in many species but lost in humans these are called hConDels, importantly the majority of these were also lost in the Neanderthal genome. Only one of the found hConDels was a protein coding region, all the others were shown to be regulatory elements that controlled gene expression of nearby genes which included steroid hormone receptor genes, neural function genes, hindbrain genes, cerebral cortex genes, fibronectin type-3 genes etc, which were found using Genomic Region Enrichment of Annotations Tool (GREAT). These 'H ConDel' include a region near GADD45G, an enhancer region mutated in humans. GADD45G is related to neural stem cell apoptosis. Our mutation allows us to not enhance GADD45G, so we are able to have increased proliferation in our SVZ and create bigger brains compared to mice and chimps. Variants of Abnormal Spindle-like, Microcephaly-associated (ASPM) control neural progenitor proliferation and have been associated with language and mutations can cause microcephaly, showing the link between brain size and language. Mcp1 transferred into chimps (7 amino acid change that causes it to be more transcriptionally active in humans) saw reduction of myelination and CNS immaturity. Mutation in humans causes microcephaly so maybe it is related to increasing NSC progenitor pool. Going back to hConDels the fact that there was a deletion event of a hConDel around an androgen receptor on the X chromosome means we don't have penis spines, which are conserved in chimpanzees and mice, I would argue to be a minorly contributing factor to our massively expanding population over chimps. Brain size has also been linked to Human Accelerated Regions (HARs), which are regions where expression of a gene has been enhanced in humans. Analyses for DNase1 hypersensitive regions suggest that most HARs function as enhancers and HiC and 3CC demonstrated associations between these regions and genes controlling brain size. HAR1A and HAR2B are HARs that are active during brain development. These unique HARs may explain the increase in growth velocity and volume in human embryonic brains vs chimps which, at 32 weeks, develop at a rate of 26cm³/week and 4.1cm³/week respectively. HAR1A has also been associated with auditory hallucinations when mutated, perhaps showing that this is the evolutionary cost of our larger brains. Human Accelerated Regulatory enhancer 5 up-regulates F2d8 associated with WNT/Beta-catenin signalling increasing NSC proliferation and gives us a large cortex. Thrombospondin 4 expression is increased due to HARs allowing us greater synaptic complexity

but also binds to Amyloid Beta plaques in Alzheimer's, signs of development cost. Another type of development cost was suggested by Matsuazwa from Kyoto universities primate research institute, which is that chimpanzees have a near photographic short term memory that was very useful for quick decision making for things like determining ripe vs unripe food or identifying the numbers of friends vs foe, while humans gained linguistics and abstraction. So, they suggest that humans were pushed away by these chips because of their ability to quickly combat or run from whatever they need to. What we also need to remember is that hConDels did not only give us bigger brains but also affected our steroid hormones, reducing our body size and made us generally weaker as well, but we gained our big heads and language. Language has been associated with FOXP2 a transcription factor which is very highly conserved, chimps and gorillas and macaques only have 1 amino acid difference compared to mice, but we have 2 amino acid changes. The evidence that these amino acid changes allowed us to speak are knock out mice that cannot squeak to their mothers. Other animals that have mutated this gene can also "speak" like birds which communicate to each other, which chirp and listen to each other's, similarly bats have mutations. It seems to cause changes in area of the brain (which in birds is called area X) that are linked with linguistics all from this amino acid change, and it seems that the FoxP2 in birds can be associated with vocal plasticity.

Our larger heads are at direct odds with bipedalism, our primary form of movement which is different from chimps and requires small narrower hips and therefore a narrower birth canal. This resulted in requiring our children to be born "premature", with the cognitive ability over the chimps initially. A chimp neonate has a cognitive ability of a 1-year old child we also maintain lactase persistence into adulthood, allowing the ability to digest lactose. Both of which are neotenous traits but gave us an advantage meaning that we can digest milk (e.g. dairy farming) and allowing our huge heads to develop, and our bodies to sort of "catch up", while we are growing outside our mothers.

Copy number variation also differs between us and chimps. For many genes we have more copies, which confers some advantages. Human population diversity means genes are also duplicated in certain groups like SULT1 which is higher numbers in Indian populations and may confer resistance to cancer as mutations increase cancer risk. CCL3 copy number increase causes HIV resistance. This can reflect society and culture as AMY1 amylase gene is highest in people with starchier diets. Alternative splicing differs too, we splice out Exon4 of GSTO2 allowing us better pathogen response by reduced enzyme activity. Gene duplication has also occurred in our eyes with opsin gene duplication.

Interestingly there are more than 200 alleles of Major Histocompatibility Complex genes in humans that occurred before divergence from chimps, these cells allow us to recognise cells that are not ours. Our T cells also differ chimpanzee T-cells, which have sialic acid conjugates that dampen immune response to prevent an overreaction of the immune response. The mutation in the caspase 12 gene also dampened the immune response, however it has come with several drawbacks including autoimmune diseases, multiple sclerosis, rheumatoid arthritis etc. This immune system changes likely came about with us encountering new pathogens, making it useful at the time, but potentially overcharged now.

In conclusion although the changes in the genome are small in terms of percentage they have dramatic affects, as they are more often than not in regulatory regions that can have great affect. These great affects always seem to come with some sort of trade off and one of the key things I took away from all of this was from a talk by Carroll where he raised the point that being the fittest is always conditional and precarious, acting only in the present, who knows what adaptations will come.

Discuss the evolution of the tetrapod limb from a fin to the wide variety of limb forms seen today. Include a discussion on the morphological and molecular changes that have had to evolved.

Paul Shepherd

February 9, 2023

Tetrapod translated from Greek means four feet and includes a wide variety of living and extinct animals including amphibians, reptiles, dinosaurs, birds and mammals. According to the fossil record tetrapod limbs evolved from sarcopterygian fish's lobe-fins, in the Devonian period approximately 370 million years ago, this fin-to-limb transition is a key evolutionary event that allowed life on land. Further evolution allowed for the wide variety of limb forms (or absences) seen today on swimming whales, walking humans, flying bats and slithering snakes (one of the exceptions to the four-footed tetrapod category). Those that kept their limbs generally maintain the homologous bone layout of one long bone (humerus) attached to two long bones (radius and ulna) attached to multiple (usually shorter) bones (carpals, metacarpals and phalanges). This essay will give an overview limb development before discussing this fin-to-limb transition and further specific evolution with the morphological and molecular changes that have had to evolve to result in these changes.

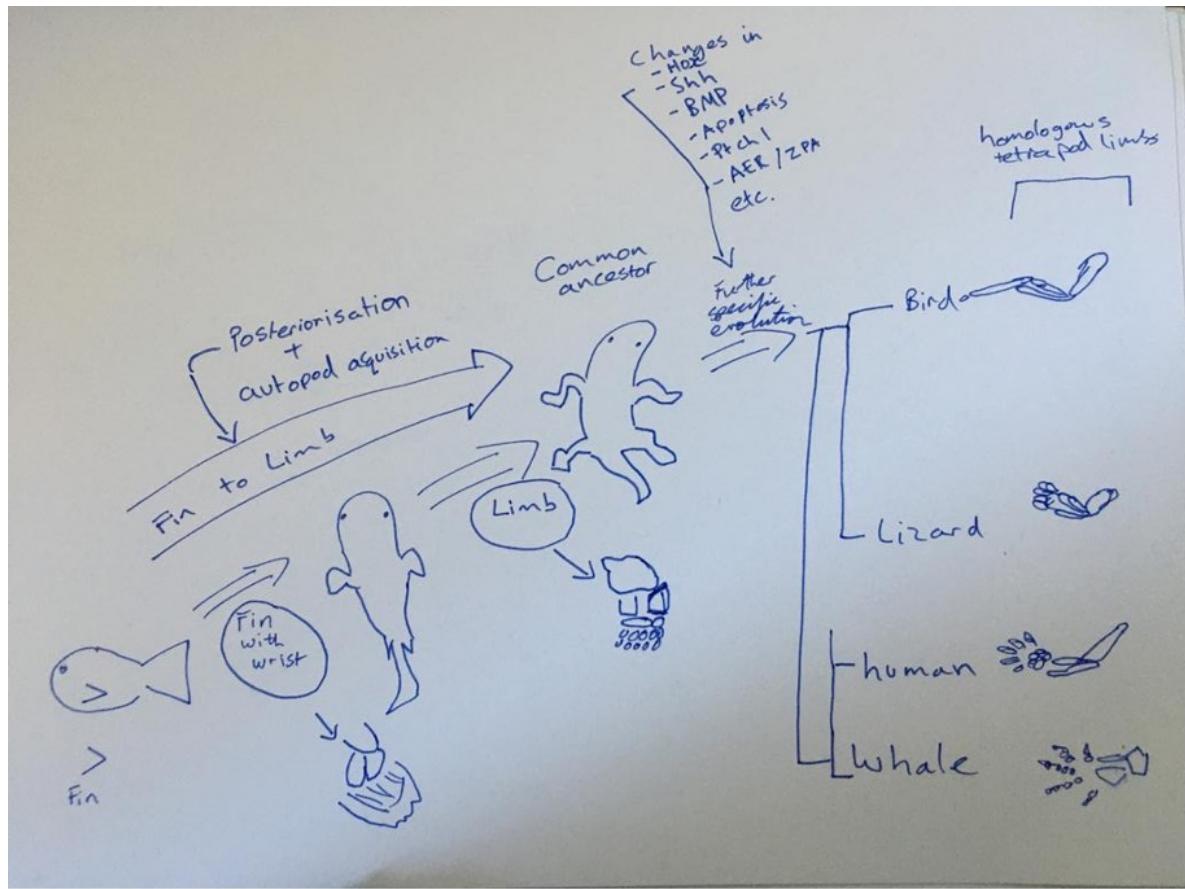


Figure 1: Fin to Limb Overview.

Limbs form in a standard way for tetrapods with the Zone of Polarising Activity (ZPA) determining the anterior-posterior axis and Apical Ectodermal Ridge (AER) determining the proximo-distal axis, these (ZPA and AER) signal to each other throughout limb development to form a fully functioning limb. The ZPA when cut out and grafted into the opposite side forms double posterior duplication showing that the ZPA is involved in digit formation. Sonic Hedgehog (shh) is the polarising factor of the ZPA and if you put a protein implant in the same location as the grafted ZPA it shows the same mirror image duplication proving that it is the molecule from the ZPA that has been shown to work in a gradient with the high concentration of shh giving rise to the little finger and low giving you your thumb. The AER is involved in proximal to distal outgrowth of the limb and this has been shown by taking the AER off during chick development. The earlier it is taken off the less distal structures are formed and the later you take it off the more distal structures are formed, the humerus is formed before the radius and ulna which are formed before the digits. We also know that fibroblast growth factors are the signalling proteins for this as beads soaked in FGF8 can replace the AER when removed to allow for normal proximal to distal outgrowth. In situ hybridisation looking at mRNA has shown shh and FGF8 locations (see sketch below) and where they overlap FGF induces shh and shh maintains FGF creating the feedback loop required for normal development. Limb positioning is determined by Hox gene expression, specifically where Hoxb-8 is strongest is where the ZPA is formed, this was shown through retinoid receptor antagonist being applied to presumptive region downregulating Hoxb-8 expression. Tbx genes are also involved in fore and hindlimb patterning with Tbx5 and Tbx4 triggering limb initiation for fore and hindlimbs respectively, by activating the Wnt/FGF signalling cascade, We do not fully understand limb development and multiple models for limb patterning have been proposed these include the; Progress Zone model, differentiation Wave-Front Model, and Turing model, but it is important to note that these molecular aspects are conserved across tetrapods.

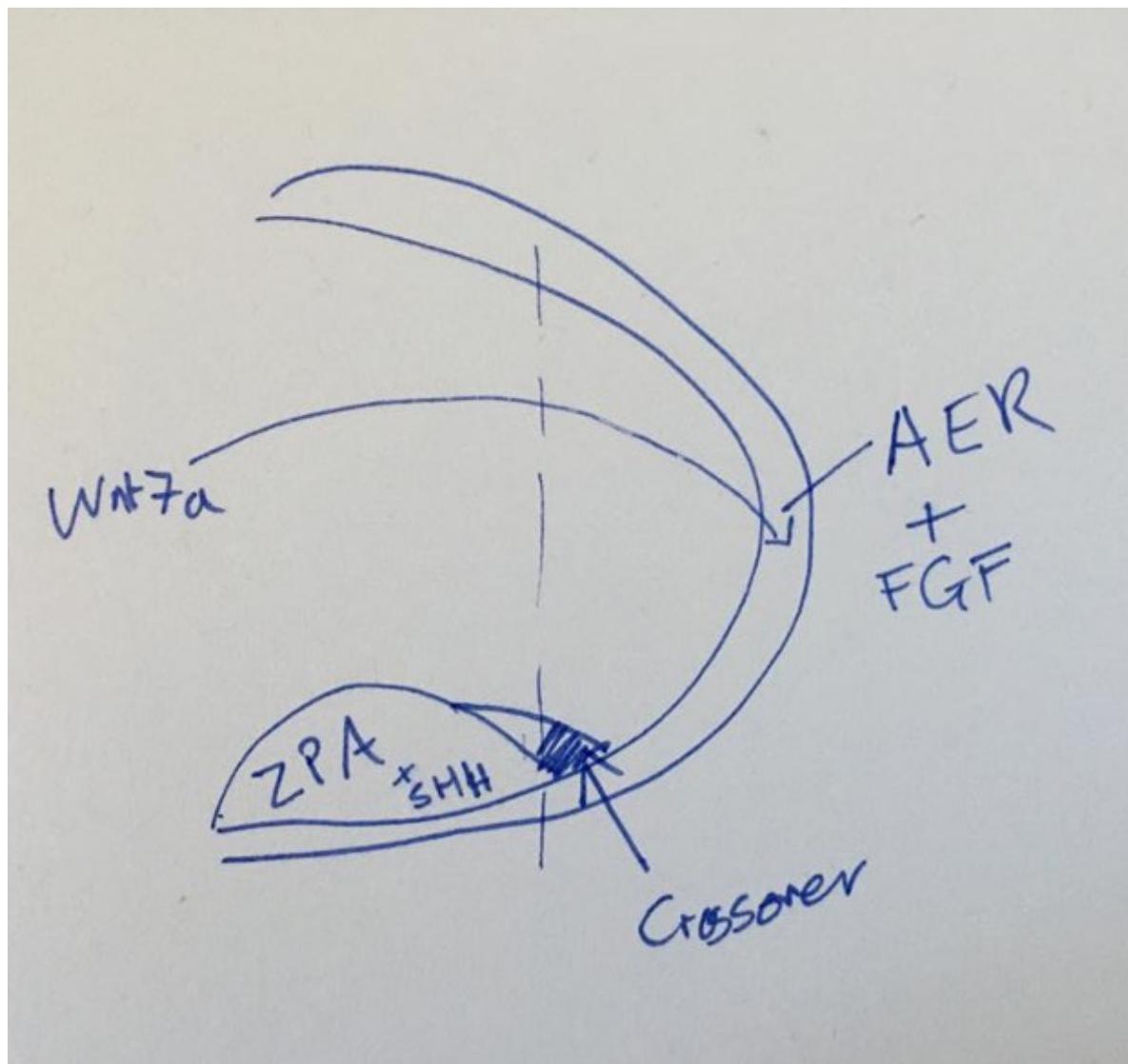


Figure 2: A figure to show the standard formation of limb development for tetrapods with the Zone of Polarising Activity (ZPA) determining the anterior-posterior axis and Apical Ectodermal Ridge (AER) determining the proximo-distal axis, these (ZPA and AER) signal to each other throughout limb development to form a fully functioning limb.

The key to fin-to-limb transition is the acquisition of the autopod, which is the most distal part of the tetrapod limb, and posteriorisation, which is the reduction of anterior structures. Fish have an AER but it folds over on itself and proliferates making the fin rays, but fish only have early phase hox gene expression while tetrapods have an early and late phase hox gene expression which allows for longer AER functioning and therefore acquisition of a hand plate. Knocking out hox genes linked to this later phase shows that it does not form these distal sections as seen in Hoxa13 and Hoxd13 knock outs where this autopod does not form. And interestingly if these are not knocked out but Hoxa11 and Hoxd11 are knocked out the hand plate has been shown to form normally but the radius and ulnar do not form correctly. Subdivision of Hoxa11 and a13 domains with the increase of shh have been shown to be linked to this acquisition of the autopod. Posteriorisation has been linked to changes in Gli3 expression, which appears to be connected to elongating the digital skeleton and defining the autopodal area as well as also being linked to Hand2 a key gene in limb bud. 5'Hoxd expression increases shh transcription therefore increasing its signalling which should also contribute to posteriorisation. And the loss of this AER folding over on itself most likely also contributes to posteriorisation as this folding has been seen in the fossil record and experiments in shark fins.

Further specific evolution come from differential growth, changes in chondrogenesis, changes in cell death, and changes in limb positioning. Varying expression of hox genes, shh, FGF etc as well as timings of these depending on the species leads to differentiation in limbs. Horses have one elongated and fused digit, and bats have elongated digits with skin flaps to allow for flight. There are different methods of reducing digits in tetrapods; these are (1) shh expression, which has been observed in skinks and the difference between 5 toed and 2 toed skinks is premature termination of shh. (2) interdigital apoptosis, which is the horse example where BMP-induced apoptosis is greatly increased in the interdigit mesenchyme when comparing horses to mice limb bud development. (3) Ptch1 expression, which when reduced due to a degenerated limb bud-specific cis-regulatory module (LRM) results in the reduction of Ptch1 expression in the mesenchyme causing a loss of asymmetry causing even toes that are artiodactyl we see in bovine and pig limbs. BMP2 expression and bmp signalling are increased in bat forelimbs in comparison to their hindlimbs and has been seen to increase cartilage proliferation. And altering BMP expression can cause or remove webbing in duck and chicken feet. Limbs can be completely removed, as in snakes (pythons) where overexpression of flank genes Hoxc-6 and Hoxc-8 can prevent limb bud initiation and when snakes are manipulated they can grow limbs, meaning they lost their ability to make limbs. Conversely you can prevent chicken limb bud induction through overexpression of these flank hox genes (hoxc-6 and hoxc-8). This idea of inducing limbs can be applied to making more digits like in polydactyl in which overexpression of SHH has been connected.

In conclusion fin-to-limb was the first evolutionary step that allowed animals to live on land as well as the water, but further evolutionary changes in the ZPA and AER as well as hox gene expression along with Ptch and bmps have allowed for the great variety of tetrapod limbs we see today.

What key experiments using amphibian embryos led to the neural default model of nervous system induction? Include a discussion of the resulting model and its potential weaknesses.

Paul Shepherd

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The neural default model of nervous system induction has been built on numerous important experiments using amphibian embryos. These experiments I would argue start with Spemann and Mangold's 1924 dorsal lip transplantation experiment, which won a Nobel prize and has been described as the most influential paper in developmental biology. I would also argue that Wolpert's line about gastrulation being the most important point in your life, applies completely to nervous system induction because without it we would not have a central nervous system and would not exist, therefore it is essential to the field of developmental biology to get an accurate understanding of nervous system induction, and identify potential weaknesses with the current model to progress. This essay will discuss amphibian embryos, early development, early developmental biology experiments leading to the neural default model, the neural default model, and its potential weaknesses.

Xenopus is a good model organism as the eggs develop very quickly, becoming a neurula within 24 hours, egg can be up to 1,500 per brood and can be visualised under the microscope, it can also easily manipulate, and the effects can be seen with a simple light microscope. But we always must question its relevance to humans with these being amphibians. These factors were large reasons for them being valuable organisms to learn about early development and was why they were being used in the 1920s, equalling the Northern crested newt (*Triturus cristatus*) shares many of these properties (which interestingly during earlier experiments two species of newt were mixed *cristatus* – non pigmented, *emphataenius* – pigmented to allow for tracing of cells). This meant that through many traditional developmental biology “cut and paste” experiments, fate maps were well established. Gastrulation involves the migration of cells from the inside to the outside causing the formation of the three germ layers; mesoderm, endoderm and ectoderm. The ectoderm eventually forms the central nervous system, peripheral nervous system and skin. Neurulation is where the ectoderm thickens to form the neural plate, which then bends by apical constriction through Rho, to fold and form the neural tube. Neurula cells are formed by convergent extension of ectodermal cells and is induced by the notochord which produces long and short range inducive signals.

Spemann and Mangold's classic experiment of grafting the dorsal lip of the blastopore onto the ventral side creates 2 heads and 2 nervous systems, and if it was taken later but still grafted into an early host the inducing signal is weaker, varying with time. Despite these only working 1/100 times it was still enough evidence to show that it was an organiser which induces ectoderm to become the nervous system. This organiser was thought to perform vertical induction and Holtfreter through the use of high salt conditions developed a gastrula, which had cells that would stay outside, but found no neural tissue as it prevented vertical induction and only allowed for planar induction. Mangold took anterior neural plate and implanted it into the cavity of the early gastrula which induced an extra head, but without a brain and transplanting the posterior gave an extra tail and spinal cord thus showing that vertical induction was needed. However, when hox genes came about, we looked at genetic markers for neural tissue Holtfreter gastrulas and found them where only planar induction was possible. So planar induction signals from the mesoderm had the potential to induce ectoderm to express neural markers. A “Keller sandwich” is where the blastopore tissue forms two embryos sandwiched under glass, they show that nervous system was still induced with homeobox marker genes (e.g. krox20 in 3rd and 5th rhombomeres) they were in the right sequence even with only planar induction. But Keller Sandwiches and exogastrula didn't have a floor plate and Keller sandwich embryos had no anterior CNS structures. Therefore, it is clear that we need both planar and vertical induction.

We wrongfully assumed for over 70 years that a single molecule was responsible for the transition of ectoderm into neural tissue, and that neural tissue is the default state for ectoderm not epidermis. The theory came from disassociating the cells of the animal cap and if reaggregation was delayed by ≥ 5 hs there was only neural tissue, while reaggregation after 1hour showed some neural markers, and immediate reaggregation causes epidermis. This meant that it is in fact the absence of intercellular signalling is required for differentiation, so with no signal they become neural, therefore they are neural by default, hence neural default model. So, it was hypothesised that the Spemann-Mangold organiser emits an antagonist into the dorsal ectoderm which blocks inductive signals and causes dorsal ectoderm to default to this neural state.

More recent experiments have been focused on discovering the molecules and receptors causing this, which with the building evidence reinforces that the neural default model is correct. Activin inhibition was shown to be present and to cause neural tissue through mRNA blocking, truncating its receptor. So, in the search for activin inhibitors began and showed that knockout noggin, chordin and follistatin together gives disrupted neural plate formation, they all block the BMP4 receptor, a receptor that is part of the TGF β family that binds to activin receptors to induce epidermis. Various experiments including ; injection of BMP4 mRNA gives ventralised embryo, grafting the BMP source onto neural plate causes a thin neural plate, adding BMP4 to disassociated animal cap giving epidermal tissue, and adding BMP4 inhibitor or mutant BMP4 receptor to animal cap cells induce neural tissue implicates them with the neural default model. But BMP antagonism alone does not induce neural tissue as FGF signalling is needed so there may be even more induces required. This unknown points to a potential weakness with the model, along with the pathways found in amphibians not working exactly in mouse/chick/zebrafish experiments, meaning that it probably does not exactly translate (as expected) to mammals. The previous weaknesses of it not being easily replicate I would argue has been overcome by modern techniques replicating it and edging this theory forward over time to the point where it has triumphed over the previously assumed epidermal default model, even if the neural default model is not fully characterised.

In conclusion the experiments using amphibian embryos leading to the neural default model of nervous system induction has been long, complex, but eventually reinforcing. Although not directly applicable to mammals, it is clear these experiments are ground-breaking and the animals have been a fantastic model organism for exploring this early vital stage of development.

Discuss the experimental evidence that neoblasts in planaria flatworms are pluripotent and how this property is relevant (or not) to their ability to regenerate missing tissues.

Paul Shepherd

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While there are many nature tv programs focused on apex predators, I would argue that they should be making more on apex regenerators, not only is it where Hollywood is going with movies like Deadpool, it is more importantly where research is going, as it human tissue regeneration is becoming more of an achievable goal, as opposed to science fiction. Planaria I would argue are a candidate for being an apex regenerator, and their cells behind their ability to regenerate are called neoblasts. Gimmicks aside, the better we understand these neoblasts the closer we get to regenerating human tissues. This essay will describe planaria and discuss the experimental evidence that their neoblasts are what make them pluripotent and how this property is relevant to their ability to regenerate missing tissues.

Planaria are non-parasitic aquatic flatworms with bilateral symmetry and 3 germ layers. There are two main types used in research in labs all around the world these are; *Dugesia Japonica*, and *Schmidtea mediterranea* and all of them are clonally related as they can reproduce asexually, this coupled with the fact that they can also reproduce sexually making them useful tools. Other aspects that make them useful model organism is that they similarly have a brain, spinal cord (sort of), a pharynx, a gut and they express homologs of important vertebrate genes, they retain 13 Hox genes though they aren't clustered, and only 5/13 contribute to axial patterning. But most importantly they have the ability to regenerate and are considered immortal. They have been studied since the 1800s, one of the most epic observations was done by H. Randolph in 1897, that showed that she could cut a planaria into pieces as small as 1/279th of a worm and they would regrow. This regenerative capacity comes from the neoblast, these are stem cells that make up 20-30 percent of cells are located throughout the organism, apart from at the tip of the head (anterior to the eyes) or in the pharynx, this suggests that they are very important for the organism. Neoblasts have been observed to regulate the organism's size as after eating, neoblasts become mitotic and cause growth, and in starvation neoblasts die back, reducing the size of the organism "de grow". This implication with the fact that neoblasts have been shown to have very little cytoplasm with the majority of the cell being nucleus, points to the fact that this cell has not much other functions like moving around or producing functional proteins etc, implicating their sole job could be to mitotically divide. Neoblasts were shown to be the only mitotic cells in planaria using Smedwi-1 and phospho-histone H3 (H3P), so if they are the only mitotic cell and an organism can regenerate all the tissues after being cut down to 279th of its original size this strongly suggests that neoblasts are pluripotent.

This was studied using radiation as in response to radiation, worms de-grow as they are unable to replace losing cells. At lethal doses (6000 rads) neoblasts cannot repopulate the cells, indicating that neoblasts have a homeostatic function to maintain worm size and function. Interestingly this is dose dependent so the more radiation the less neoblasts are able to replace dead and missing cells. At 1750 rads neoblasts begin to reappear mainly between the eyes and the pharynx. The cluster size increases while frequency of the clusters decreases indicating that these cells are dividing. And this mitosis recovers over time then plateaus after 2 weeks showing that it is under control and not uncontrolled cancer. Then in 2011 to prove neoblast pluripotency they inserted of single neoblasts (from an asexual colony) into fatally irradiated worms (sexual colony) to show that it can cause survival. Interestingly, using a single neoblast from asexual worms into lethally irradiated sexual worms can prompt not only regeneration but loss of sexual characteristics. Given that germ line neoblasts express nanos, perhaps asexual neoblasts cannot express this. Importantly only 20 percent of single neoblasts injected worms

had colonies form. In the best conditions, which included using neoblasts with protrusions, 75 percent of worms could be rescued with cluster formation. So, with unknown specific type of protruding neoblast a larger percentage survive compared to neoblasts without this protrusion (which have shown to express different markers through FACs indicating they are fundamentally different cells), showing that some cells neoblasts are better than others, and hence showed that neoblasts are pluripotent but not all of them are, suggesting that these protruding neoblasts are the pluripotent ones and others are not.

The evidence that neoblasts control regeneration is shown that in “cut up” and fatally irradiated injected neoblasts can save the planaria. Also damage at the very tip where there are no neoblasts normally show that neoblasts can actually migrate across to (possibly being pushed there by other cells), colonise and regenerate the site of the injury. There, cells have been shown to divide to produce nerve cells or differentiate for tissue repair. Wound response has been characterised by 2 phases and neoblasts are central to this, where neoblasts migrate to the damaged region. Phase 1 is a generic response and mitosis of neoblasts throughout the planaria, and phase 2 is where they actually detect missing tissue, a signal that causes neoblasts to accumulate in the damaged area, and cells divide while some neoblasts fall out of their stem cell state to initiate blastema formation. So, some neoblasts act in different ways and neoblasts have been shown to come in three categories though no direct homology to transit amplifying cells has yet to be found. Category 1 neoblasts are formed at the middle of the worm and have tentative pluripotency marker piwi/smedwi. Category 2 neoblasts are early division progeny in the middle of the worm and express nb21.113 and inx11 while category 3 are late progeny which are towards the periphery that express Agat. Unique populations of neoblasts may exist, like FoxA at pharynx and Ovo+ at eyes. This shows that not all neoblasts are created equal but depending on what structure they are regenerating will have different roles, whether that be just creating more cells or creating specific cells to that new repaired tissue.

In conclusion neoblasts in planaria are varied in their pluripotency as there are different subdivisions of neoblasts, some can be considered totally pluripotent some other categories are not. This pluripotency is essential when considering the scale, it can regenerate from (1/279) but the full extent of pluripotency might not always be necessary when dealing with minor damage to the periphery in which case lesser neoblasts could repair the structures as it might not need as wide array of cell types. Through understanding these varied neoblasts we can use this knowledge to make breakthroughs in human regeneration, and just as we have become apex predators through weapon engineering, we can become apex regenerators through genetic engineering (ethics permitting).

What is ‘gene therapy’? Using examples, describe how gene therapy can be achieved, when it is appropriate, and outline some of the key safety considerations.

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Gene therapy is using genetic material (DNA/RNA) to treat disease. There are numerous methods to achieve gene therapy that can be temporary (in the case of somatic cell gene therapy) or permanent (in the case of germline gene therapy), this distinction comes with its own safety and ethical concerns. The methods for actually getting the therapeutic genetic material into cells whether they be viral or non-viral, also come with their own safety concerns. But the actual editing technology of CRISPR and its subsequent adaptation to make it safer has increased its viability and with it brought more investment and research making gene therapy an exploding area. It can be done on individuals with a disease, or during the first stages of development (at a one or 2 cell stage) fundamentally changing our genetic code. The scientific capability appears to be getting ahead of the ethics (and possibly legal systems) which in itself brings safety concerns, alongside the debates about what conditions should these techniques be used on? This short essay will not have time to examine this topic fully but will explore a few methods of how gene therapy can be performed, with comments on safety considerations, and make comments on which diseases and circumstances it could be appropriate.

To actually get the DNA into the host cell this can be achieved through viral or non-viral methods. In the case of viral the DNA is packaged into a virus wither it be adenoviruses, adneo-associated viruses or lentiviruses so depending on your goal you can use anyone of these. Adenoviruses (AAVs) can put DNA in a non-permanent manner as the viruses are eventually eliminated by the innate immune system and can package up to 7.5kbps. Adeno-associated viruses are smaller but are non-pathogenic. AAVs integrate into a site on chromosome 19 but this function can be altered out and has been used against haemophilia, lipoprotein lipase deficiency, and retinal dystrophy (by AAV delivering RPE specific protein cDNA into the subretinal space). Lentiviruses are retroviruses with an RNA genome which gets reverse transcribed to DNA and can be transcribed, most of these are HIV derivatives engineered to be safe. So, it can integrate DNA long term and has been used to treat things like x-linked adrenoleukodystrophy. Retroviral therapy has also been used to treat successfully treat SCID by transfecting healthy adenosine deaminase cDNA into patient T-cells before putting them back into patients, these transduced lymphocytes survived long term. But viral based treatment of SCID has also cause failure of B and T cell function and caused cancer and death through off site effects as it integrated close to an oncogene causing upregulation. Also, Jesse Gelsinger, must be mentioned when considering safety considerations with regards to gene therapy and viruses as he unnecessarily died from it. He had a mild/mosaic form of ornithine transcarbamylase deficiency, which affects urea processing and causes ammonia accumulation and in severe cases is fatal at birth, but his milder form just meant a manageable restricted diet and medication. In his case he had an immune reaction to the virus, which lead to multiple fatal organ failure and brain damage, and it turned out that lead investigators had overlooked red-flag symptoms in animal models because they had financial interest in the company that aimed to make profits from this research. Also, viruses have the risk of crossing over in the sense that if someone had normal and untreated HIV/coronavirus/flu then had gene therapy via a viral vector and these viruses interacted and adapted it could become a contagious genome editing virus that unintendedly changes human genomes globally.

Arguably safer vectors for gene therapy like liposomes can be used to deliver DNA like a “trojan horse” with hydrophobic and hydrophilic residues that bound to DNA can it across cell membranes. This method was used with limited degrees of success in 2015 to safely treat 78 cystic fibrosis patients. Nanoparticles can also be used in the form of synthetic polymers which form nanoparticles when

mixed with DNA that can be endocytosed thus delivering gene therapy. Other methods on non-viral like injection of naked DNA, gene guns, magnetofection, sonoporation and electroporation can be used but could have unseen implication so it would be important to extensively test and perfect methods.

Gene therapy can use short interfering or short hair pin RNA to cause mRNA degradation by binding and direction of RNA induced silencing complex to cleave the target mRNA. SiRNA is short (22bp) and double stranded RNA of the gene of interest, which is then digested by DICER and guides RISC to the target, if the RNA is too large it can cause an antiviral response, but both Si and Sh RNA have been used in Huntington's treatment. Facioscapulohumeral muscular dystrophy was reversed by targeting FRGI with shRNA (delivered by AAVs) in FGRI overexpressing mice. Gene therapy can also use CRISPR/Cas9 DNA "cut and paste" technology, which has been modified to reduce unwanted off target effects. This could be used on the over 32,000 SNPs identified as pathogenic. With this we have unprecedented capability to fundamentally treat/eliminate genetic diseases, this calls into question which diseases, I would argue with all the risks associated with it we should be focused on treating only the most severe otherwise untreatable diseases through these methods, as diseases that can be managed through other means does not warrant the risk. I would also argue that changing the human genome during development not only is open to unexpected risks, but a slippery slope as to which diseases you eliminate, as it could start with severe and fatal SCID, then lead to the elimination of the manageable form that Gelsinger had, to then achondroplasia, to eye colour and so on. CRISPR babies have already been attempted with He Jiankui attempting to make HIV resistant children was met with staunch debate around what could arguably be an important cause, but I believe the reaction towards it was not positive. And if there are huge reservations about changing mice to make them resistant to and eliminate tick borne diseases (in America) due to the ecological impact, I cannot imagine we are anywhere near making germline changes in humans even if it is just for resistance.

The safety concerns around gene therapy and their potential negative effects through unseen immune responses (or unexpected gene interactions or unintended viral spread) but also safety considerations about those developing or giving the treatment. Gelsinger showed that mislead financial incentives, just like mislead ideology in WW2 allowed doctors to do unnecessary and fatal experiments in the name of progress. I am not saying that this was equivalent to the atrocities of Nazi doctors, but I would argue that taking a do no harm oath is not enough to stop misguided experimentation with gene therapy. Currently a major safety consideration is that we lack a good enough legal and monitoring framework because currently depending on where you live this technology can be bought by anyone on the internet. And should someone want to permanently change germline cells they can and ultimately play god, they can, and it is not only limited to researchers who took the Hippocratic oath and those who didn't it is anyone. The advocates in America argue for this to be free to use within the "biohacker" community as they believe that if everyone has it no single group can gain the upper hand with "super humans" is misguided and dangerous for the reasons above, and I would argue giving everyone access to it would only increase the risks of future problems/atrocities.

Discuss how intrinsic and extrinsic factors modulate (i.e. change) transmitter release at the neuromuscular junction.

Paul Shepherd

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Development of our nervous system is a lifelong process, synapses are not hardwired we constantly adapt to learn/perform new tasks, so we need to adapt our dynamic synapses to become stronger or weaker in certain ways. Such as; through changing transmitter release, through intrinsic factors that are integral to the terminal, or extrinsic factors which come from outside, external to the terminal. Abnormalities in neurotransmission are associated with autoimmune disorders (Lambert-Eaton myasthenic syndrome (LEMS) and myasthenia gravis) and genetic diseases (congenital myasthenic syndromes (CMS)) which can be through faulty neuromuscular junctions (NMJ). The NMJ is a simple model for synaptic transmission as it is, in theory, a single nerve terminal on a single cell, which can be used to understand transmitter release and the target for therapeutics. This essay will first give an overview of neurotransmission at the NMJ before discussing intrinsic factors, including; activity, vesicle content and peptide co-transmitters, as well as extrinsic factors, including; noradrenaline, adrenaline, target size, muscle fibre type, target abundance and disease.

Neuromuscular junctions have a presynaptic neuron, a synaptic cleft and a postsynaptic cell. When an action potential reaches the end of the presynaptic axon it reaches the axon terminal which is a region specialised to releasing neurotransmitter. Here voltage gated sodium channels open to depolarise the terminal, causing the voltage gated calcium channels to open in response to that depolarisation, it is these calcium trigger release of the neurotransmitter from vesicles through exocytosis, which is then received by specific receptors on the postsynaptic cell. In the NMJ this neurotransmitter is acetylcholine, which binds to the postsynaptic cell acetylcholine receptors that opens a ligand gated non-specific ion channel that allows predominantly sodium in, potassium out and calcium in. This causes a depolarisation in the muscle cell, which in a functioning cell will be large enough to reach threshold to trigger voltage gated sodium channels generating the action potential in the muscle cell which is the first step leading to muscle contraction. The neurotransmitter and membrane are recycled replenishing the vesicle pool. Neuromodulation can be considered changing the number of vesicles released per action potential, which is the quantal content (QC).

An intrinsic factor that impacts QC is the activity that the terminal experiences in that what it is doing now will influence what the terminal does next. This can be seen with the NMJ in a transmission assay, facilitation is where a first/earlier action potential makes a greater release of vesicles for a subsequent action potential meaning, the postsynaptic response gets bigger after the low initial QC, this has been shown experimentally by reducing calcium and therefore the probability of release. Depression is where a first/earlier action potential causes subsequent action potential to release less vesicles, the post synaptic response gets smaller after the high initial QC. This is influenced by the number of vesicles in the presynaptic ready releasable pool (RRP), neurons which cause facilitation have a large RRP while those causing depression have a smaller RRP, this occurs in a very short time scale (milliseconds). Later is post-tetanic potentiation, which is due to mitochondria soaking up the calcium during activity, which is slowly released, activating kinases, which phosphorylate synapsin, to release the reserve pooled vesicles to the RRP allowing for more transmitter release with the next action potential. There is even later long-term potentiation (LTP) where calcium activates genes to permanently reinforce the synapse.

Another intrinsic factor is vesicle content and feedback, as they can contain Acetylcholine and ATP. Ach acts on M1 muscarinic receptors and M2 muscarinic receptors that are there to determine and influence what is going on in the synapse, which are not fully understood but both influence QC. M1 activates Protein Kinase C which increases Ach release, and M2 activates Protein Kinase A

which inhibits ACh release so depending on the amount, locations and interactions of these receptors will influence the neuronal signalling. ATP also causes negative feedback by it breaking down to adenosine which is then influences the Adenosine A1 receptor which acts on Protein Kinase A to decrease neurotransmitter release.

Peptide co transmitters inside the terminal also influence transmission. Unlike the vesicles previously talked about which were small and clear, there are also large dense core vesicles these are fewer and need more calcium stimulation to be released need to move to be released. These large dense core vesicles contain calcitonin gene related peptide (CGRP), which increase cAMP in muscles therefore increasing contraction. They also contain vasoactive intestinal polypeptide (VIP) which influences release, they also contain agargin which will also influence the synapse.

Extrinsic factors like noradrenaline/norepinephrine from the closely situated autonomic nervous system specifically the sympathetic NS this through the alpha2 receptor increases transmitter release through a system we do not fully understand. Also, adrenaline/epinephrine from the endocrine system through the beta2 receptor makes the postsynaptic cell more sensitive to depolarisation as it acts on the potassium channels to reduce leak, increasing the post synaptic response. Target size, as in how big the target cell is, so in the case of NMJ how big the muscle fibre diameter is. Small fibres have a high input resistance because they cannot have as much leak as they have a smaller surface area so need less transmitter (Ach) to drive to threshold and cause a response. Large fibres or larger target cells have more leak due to more surface area and require more transmitter release to reach threshold. On the other side the small terminals will release less vesicles and therefore neurotransmitter as they will have fewer active zones and therefore a low quantal content. Conversely larger terminals have more active zone due to their size and therefore increase QC. So, depending on what you want you can change the sizes of either size accordingly, so long term changes might mean the synapse junction would grow to fulfil the greater transmitter release.

Muscle fibre type reflects the pattern of use, so fast muscle fibres have a high quantal content, while slow muscle fibres have a low quantal content. This has been seen in the EDL and soleus where initial QC, vesicle pool size and recycle time adapt accordingly, so the fast EDL has a high initial QC, but a low vesicle pool and long recycle time, and the slow soleus has a low initial QC, large vesicle pool and short recycle time allowing it to work for longer periods of time. Target abundance, as in the number of muscle fibres innervated in the case of the NMJ, also influences transmitter release as if a single neuron is innovating 5 muscle fibres QC per terminal decrease, but if it innovating less (2 fibres) QC per terminal increases shown by the reinnervation/damage protocol and is thought to be linked to the amount of metabolic stress.

Disease also influences transmitter release. LEMS linked to lung cancer blocks calcium channels responsible to transmitter release. Myasthenia gravis is where antibodies attack Ach receptors which reduces the synapse efficiency, the presynaptic neuron tries to compensate with higher QC. Just as disease can influence any of the receptors involved in these medical therapies can interact with these receptors to change neurotransmitter release.

In conclusion, whether it be learning/performing a new skill physical or mental our nervous system must adapt each neuronal junction according to its given task. This can be done intrinsically or extrinsically through varying the size of the; initial transmitter release, reserve pools (and their locations), junction as well as the position and numbers of functioning receptors that influence transmission.

Discuss how proteomic tools can be used to identify and understand a specific disease in a given population.

Paul Shepherd

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From the start of our scientific education we are told proteins are the building blocks of life, so we know they are a wide range of complex molecules that are critical for the normal function of our cells and body. All the proteins being expressed at a given time in a particular cell, tissue, or organism is known as the proteome. Protein expression changes can result in very different cellular actions and upregulation or downregulation of certain proteins can cause and show disease. Expression proteomics can identify the level of proteins in two or more states like in disease vs normal, so we can find proteins that are associated with disease, thereby diagnosing or finding targets to treat disease. Functional proteomics allows for the understanding of a given protein's function through identifying interactions between proteins, which in the case of diseases means we can understand the actual reasons behind a disease. This essay will discuss how proteomic tools including 2D gel electrophoresis, high performance liquid chromatography (HPLC), matrix-assisted laser desorption/ionisation – time of flight mass spectrometry (MALDI-TOF MS), liquid chromatography and tandem mass spectrometry (LC-MS/MS), and protein microarrays to identify and understand a disease in a given population using Alzheimer's disease (AD) as an example.

Two-dimensional gel electrophoresis separates proteins by charge and mass, and thus identifies proteins by differences in these. It involves putting an SDS-PAGE gel onto the other end of another gel running perpendicular based on charge. SDS has been stripped off the protein so it goes back to its original charge. It can be used to assess changes to protein levels in response to certain drugs or identify proteins that cause harmful effects. In the context of AD a study that took 15 AD temporal cortex brain samples and compared it to 15 control samples, found 28 proteins that were significantly decreased in AD brains, 5 proteins that were significantly increased in AD brains, and 9 proteins that were only found in AD brain samples, which with now identified will allow for targeted future research of the pathogenic protein changes. HPLC allows for the precise separation of similar proteins, by pumping a given sample through a tube filled with beads/gels, separating them by charge or mass or both or a given property such as phosphorylation. It has been used to screen for sickle cell anaemia by identifying haemoglobin variants. In the context of AD HPLC coupled with Tandem Mass Spectrometry can detect Amyloid beta 1-40 peptide in blood samples to incredible accuracy which has been previously implicated with the development of AD, and can therefore open it up as a possible biomarker for the detection and diagnosis or mark it as a significant molecule to research and understand the process of AD opening up possible treatment strategies.

MALDI-TOF MS gives mass : charge ratios of samples as the samples on a metal plate are hit with a laser, vaporised and charged ions are accelerated along an electrical gradient and timed, the larger particles hit the detector later and usually the more positively charged the more it is pulled in X direction allowing identification of proteins. This can again be used to identify biomarkers either in blood or cerebrospinal fluid that could catch the disease early, or identify proteins implicated with a given disease. In the case of AD, MALDI-TOF MS with tandem mass spectrometry (see paragraph below) has been used to study and characterise proteins isolated from senile plaques (a structure in AD brains), assisting with the understanding of the formation and growth of these plaques, which opens up areas of research for potential future therapies. LC-MS/MS uses liquid chromatography to separate them then mass spectrometry to identify them and tandem mass spectrometry lets you pick a substance and use only that mass for mass spectrometry again as it passes the sample through argon gas and they are detected and separated by individual amino acid differences. In the case of AD, ultra-performance LC-MS has been used to identify 9 potential biomarkers for AD when comparing the plasma metabolic profiling of AD vs control, it also showed a correlation between the severity of AD.

This was then narrowed down further to potential LPCs, sphingosine and tryptophan, which could be used to catch AD early and start treatment.

Protein microarrays use antibodies on the array and can use proteins that bind to other proteins to find binding partners or protein complexes. In the case of AD this has been used to identify AD blood biomarkers with a high sensitivity (96.0 percent) and specificity (92.5 percent) distinguishing it clearly from other cancers that share overlapping markers. This again potentially opening up opportunities for early diagnosis and treatment. In conclusion the above has described how different proteomic tools can be used for the identification and understanding of a specific disease by comparing samples from different populations. This essay used AD as an example for each of the techniques, but this could just as easily be almost any other disease pretty much always will change protein expression. We all simply have to love these building blocks of life/disease and the proteomic techniques that have saved lives.

Müller glia in Retinal Disease and Regeneration (498 word presentation summary).

Paul Shepherd

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With the 962million people over 60 is expected to double by 2050 (United Nations 2017) and the 488million diabetics expected to double by 2030 (WHO 2016) the global population is getting older and increasingly diabetic. As aging is strongly associated with the neural retina separating from the retinal pigment epithelium known as retinal detachment (Chang et al., 1995, Mitry et al., 2009, Van de Put et al., 2012). Plus, the hyperglycaemia from diabetes causes Müller Glia (MG) and capillary cell death, resulting in diabetic retinopathy, the leading cause of vision loss among working-age adults (Zong et al., 2010, Lee et al., 2015, Zeng et al., 2016) retinal degeneration is increasing. But MG are being researched as they show latent stem cell properties so could drive human retinal regeneration (Fischer and Reh 2001).

MG span the retina from the inner to the outer limiting membranes providing structural, homeostatic and metabolic support of retinal neurons (Reichenbach and Bringmann, 2013). But how MG arise is an active area of research, retinal differentiation is stimulated by a wave of Sonic Hedgehog (Shh) (Jarman, 2000) and MG are the last of the 7 specialised retina cell types to arise from retinal progenitor cells with Zeb2, Hes5 and Sox9 transcription factors involvement (Xiang 2012, Wei, et al. 2019). The determination of cell fate is not entirely understood with 3 proposed models, with each losing pluripotency in mammals with time (Reese 2011, He et al., 2012, Lamb, 2013). However cold-blooded animals such as zebrafish MG can maintain this pluripotency giving rise to all types of retinal neurons without injury trigger but for warm-blooded mammals injury is required (Goldman 2014).

Gliosis is the term used for this process of MG reacting to retinal damage, where damaged cells releasing ADP, TNF-alpha, Wnts and growth factors (GF) cause MG to prevent tissue damage by releasing antioxidants and neurotrophic factors. While microglia promote apoptosis through cytokines and the removal of neurons via phagocytosis (Silverman and Wong, 2018), in mammals gliosis ends with the formation of a glial scar that inhibits regeneration. However, in zebrafish GF, Wnt and cytokine signals cause MG reprogramming resulting in the following steps; (1) MG de-differentiation into retinal progenitors, (2) proliferation, (3) neural differentiation of progeny, and (4) integration into retinal circuitry (Goldman 2014). This is mediated by Increasing SHH, Notch decreasing allowing dedifferentiation influencing Stat3, which increases Ascl1a, that is linked to pluripotency factor Lin28, which when increased causes a decrease in let7. If we can replicate this in humans, we can drive MG led retinal regeneration.

To access the neurogenic potential of MG in mammals it's necessary to prevent the standard response to injury which results in the upregulation of reactive gliosis, and downregulation of proliferative genes. This may require decreasing the usually increased Notch upon injury to mimic the zebrafish response. Shh can stimulate mammalian injured and uninjured MG to proliferate (Wan et al., 2007) and p27KIP1 will also need to be controlled as it is an important factor to initiate the proliferative response. Ascl1/Mash1 is vital for neural differentiation and outgrowth, overexpression in injured young mice showed retinal regeneration (Ueki et al., 2015), along with histone deacetylase inhibition shows limited regeneration in adult mice (Jorstad et al., 2017). MicroRNAs are also being researched with miRNA-124 supressing miRNA let-7 inducing Ascl1 that results in 40 percent of MG to reprogrammed neuronal cell types (Wohl et al., 2019). Despite these advancements more research is needed before achieving therapeutic human retinal regeneration.

References

- Chang, C.J., Lai, W.W., Edward, D.P. Tso, M.O. (1995). Apoptotic photoreceptor cell death after traumatic retinal detachment in humans. *Archives of ophthalmology*. 113, 880-886
- Fischer AK Reh TA. (2001). Müller glia are a potential source of neural regeneration in the postnatal chicken retina. *Nature Neurosci*. 4:247-252.
- He, J., Zhang, G., Almeida, A., Cayouette, M., Simons, B. and Harris, W. (2012). How Variable Clones Build an Invariant Retina. *Neuron*, 75(5), pp.786-798.
- Goldman D. Müller glial cell reprogramming and retina regeneration. *Nat Rev Neurosci* [Internet]. 2014 Jul;15(7):431–42. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/24894585>
- Jarman, A. (2000). Developmental genetics: Vertebrates and insects see eye to eye. *Current Biology*, 10(23), pp.R857-R859.
- Jorstad, N., Wilken, M., Grimes, W., Wohl, S., VandenBosch, L., Yoshimatsu, T., Wong, R., Rieke, F. and Reh, T. (2017). Stimulation of functional neuronal regeneration from Müller glia in adult mice. *Nature*, 548(7665), pp.103-107.
- Lamb, T. (2013). Evolution of phototransduction, vertebrate photoreceptors and retina. *Progress in Retinal and Eye Research*, 36, pp.52-119.
- Lee, R., Wong, T. and Sabanayagam, C. (2015). Epidemiology of diabetic retinopathy, diabetic macular edema and related vision loss. *Eye and Vision*, 2(1).
- Mitry, D., Charteris, D., Fleck, B., Campbell, H. and Singh, J. (2009). The epidemiology of rhegmatogenous retinal detachment: geographical variation and clinical associations. *British Journal of Ophthalmology*, 94(6), pp.678-684.
- Reese BE (2011). Development of the retina and optic pathway. *Vision Research* 51, 613-632. doi:10.1016/j.visres.2010.07.010
- Reichenbach, A. and Bringmann, A. (2013). New functions of Müller cells. *Glia*, 61(5), pp.651-678.
- Silverman, S. and Wong, W. (2018). Microglia in the Retina: Roles in Development, Maturity, and Disease. *Annual Review of Vision Science*, 4(1), pp.45-77.
- Ueki, Y., Wilken, M., Cox, K., Chipman, L., Jorstad, N., Sternhagen, K., Simic, M., Ullom, K., Nakafuku, M. and Reh, T. (2015). Transgenic expression of the proneural transcription factor Ascl1 in Müller glia stimulates retinal regeneration in young mice. *Proceedings of the National Academy of Sciences*, 112(44), pp.13717-13722.
- United Nations (2017). World Population Prospects: the 2017 Revision. (01/11/2019) https://www.un.org/en/development/desa/population/publications/pdf/ageing/WPA2017_HIGHLIGHTS.pdf
- Van de Put, M., Hooymans, J. and Los, L. (2013). The Incidence of Rhegmatogenous Retinal Detachment in The Netherlands. *Ophthalmology*, 120(3), pp.616-622.
- Wan, J., Zheng, H., Xiao, H., She, Z. and Zhou, G. (2007). Sonic hedgehog promotes stem-cell potential of Müller glia in the mammalian retina. *Biochemical and Biophysical Research Communications*, 363(2), pp.347-354.
- WHO (2016). Global Report on diabetes 2016. (Accessed 01/11/2019) <https://www.who.int/diabetes/global-report/en/>
- Wohl, S., Hooper, M. and Reh, T. (2019). MicroRNAs miR-25, let-7 and miR-124 regulate the neurogenic potential of Müller glia in mice. *Development*, 146(17), p.dev179556.
- Zeng, K., Yang, N., Wang, D., Li, S., Ming, J., Wang, J., Yu, X., Song, Y., Zhou, X. and Yang, Y., 2016. Resveratrol prevents retinal dysfunction by regulating glutamate transporters, glutamine synthetase expression and activity in diabetic retina. *Neurochemical research*, 41(5), pp.1050-1064.
- Zong, H., Ward, M., Madden, A., Yong, P.H., Limb, G.A., Curtis, T.M. and Stitt, A.W., 2010. Hyperglycaemia-induced pro-inflammatory responses by retinal Müller glia are regulated by the receptor for advanced glycation end-products (RAGE). *Diabetologia*, 53(12), pp.2656-2666.