CGE-01027-2017

Genetic pathways of split hand-foot malformation

Kantaputra, Piranit

Reviewer: 1

Comments to the Author

Subject: Genetic Pathways of Split Hand-Foot Malformation

The review is generally well written by providing the relevant information. Following minor changes may be considered to strengthen the review article.

1.   Page 6, line 23-24: Reference Ullah et al 2016 is missing from the list of references. The link for the above mentioned paper: http://onlinelibrary.wiley.com/doi/10.1111/ped.13023/full

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4.   The authors have not mentioned the SHFM related gene BHLHA9 that has been shown to be relevant to AER and phalangeal reduction (Kataok et al 2017; Khan et al 2017). The authors should discuss its role in this paper.

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5.   On page 11, lane 30-31" The authors assert that the down-regulation of FGF8 in AER is the cause of SHFM. About relevant SHFM related gene BHLHA9, it has been shown that the up-regulation of FGF8 in AER may be the cause of the disease (Kataok et al 2017; Klopocki et al 2011). The authors should also take this difference into account for more comprehensive interpretation for SHFM pathogenesis.

6.   The authors should discuss the molecular link between SHFM-related genes and the process of chondrogenesis in limb bud in their discussion on the onset of SHFM phenotype in limb formation. The link of EPS15L1 in chondrogenesis should be considered. EPS15L1 related receptor EGFR and its pathway has important role in chondrogenesis (Yoon et al 2000).

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7.   The authors have focused on the role of FGF8 in SHFM. Accordingly they should discuss its role at cellular level in AER elaborating pathogenesis in the light of available research data. One relevant paper [Gros et al 2010] has discussed effect of the FGF8 on the mobility of cells in the limb bud.

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9.   For common readers, it would be more useful if the authors present clinical features result from mutations in different SHFM genes. In the table mutations reported so far can also be added.

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The intent of this review is to highlight some key pathways relevant for the comprehension of the congenital limb malformation known as SHFM. The authors combine human genetics with mouse developmental biology to push the concept that FGFs are the key signals that (when perturbed) would cause misorganization of the AER, and that Wnt signaling is upstream of FGFs.

Major Comments on the text

A. The concepts indicated above are neither wrong nor fully correct, overall the analysis and interpretation of the available data that the author propose appear quite superficial and mis-focused. I did not find this review useful, and on some aspect it is misleading.

Consider that:

a) Wnt signalling is not ONE signal but rather a wide class of ligands/receptor systems, which are sequence-related but are implicated in nearly all developental processes, from proliferation to differentiation, from cell polarity to oriented cell division and migration, etc… WHICH of the following the authors propose ? and why ? Wnt10b and Wnt5 have been implicated in SHFM, while Wnt7b is implicated in dorso-ventral orientation, and Wnt3 is implicated in early AER indcution. WHICH of these

? Although they are all "WNTs", they activate partially distinct and partially overlapping pathway and certainly carry out distint functions.

b) The same fo the FGFs. These are potent mitogenic and patterning signals, but also orient the migration of limb mesenchymal cells. FGF8 appears to play a role in SHFM, however this notion is derived from mouse models. Hence, in the same model, the mouse limb bud, WHAT is the role of (DIRECTLY) altered FGF signaling and AER formation ? Does altered FGF signalling result in altered digit patterning ? This is particularly important, since the rest of the review deals with issues related to digit patterning.

The authors should focus on those individual Wnt and FGF ligand/receptor systems directly implicated in SHFM, and examine in depth their individual and combined roles. The issues of digit patterning and proximo-distal extension have been reviewed elsewhere and cannot be illustrated here "generically" . Each signaling molecule DIRECTLY implicated in SHFM should be re-examined in the context of known developmental processess that take place during limb development. At the end of reading the reviewe the KEY question remains : WHY the central digits are affected (as consequence of the altered Wnt and FGF signaling at the AER) while the other digits are nearly normal ? Which is to say, how can the digit patterning be unaffected while central digits are severely hypoplastic ?

B. As correctly stated, SHFM is a limb developmental defect that is closely linked to mis-function of the AER; however there is no clear evidence of an altered digit indentity. The fact that the remaining digits appear malformed (hence cannot be assigned digit number based on morphology) is of little meaning. A molecular identity should be assigned, and no study that I am aware of has examined this. On the contrary, the expression of SHH, Gremlin and HOX genes is relatively normal in the limbs of mutant mouse models models of SHFM. Therefore, the paragraph "Embryological Mechanisms of Digit Patternng" of this review is scarcely relevant, is poor of scientific content and does not contribute relevant observation or connections.

C. The same can be said about the section "Mirror Image Morphogenesis in SHFM". The section concludes that there is NO mirror image defect in SHFM, something that is expected since the phenotype is linked to altered maintenance and/or activity of the AER which form at a later time with respect to limb induction and general anterior-posterior patterning. Curiously, the authors state in the Abstract that "mirror image morphogenesis is disrupted in SHFM". Which one is true ? This appears as an evident contradiction.

D. In the Abstract as well as in the Introduction, the authors mention "mutations of FGF8", however I do not know about these mutations in human, they are not specifically cited in the reference list, and they don't appear in the literature. Please double check this information.

E. In the Abstract as well as in the Introduction, the authors maintain that since different gene mutations cause an undistinguishable phenotype, this implies that they share a common pathway. This is plausible, but not necessarily true. The indicated genes could cooperate and act in parallel – partially redundant – pathways (known for FGFs and Wnts) or they could act separately and converge onto – and thereby regulate – a common core process, yet to be identified. The authors should be cautious in their statements.

Finally, some citation are wrongly cited. The authors should double check and make sure that each references is correctly placed in the text, and really supports what the text says.

About the scheme proposed in Figure 2

Figure 2 proposes a scheme which I find quite confusing and misleading.

First of all, being a quite complex scheme, which attempts to convey a large set of informations, it needs a detailed legend, indicating the meaning of colours, arrows, etc..

Second, most importantly, the scheme mixes up human and mouse, transcription factors, signaling systems, demonstrated regulations and hypotheses. The reader is uncertain as to which arrow to follow and what is the scientific content. Having a reference reported in the box does not help the comprehension. The message(s) proposed are too many, the purpose of the scheme is unclear, and the resulting scenario is confusing. Furthermore, the text does not follow the scheme or viceversa, therefore it is not a helpful integration.

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Some examples:

1 (on the left) what connects abnormal Wnt signaling with mutations in Dlx6 ?

2 (on the left) what connects abnormal expression of DLX5 and DLX6 with Abnormal Sp6 and Sp8 expression ? Has this been demonstrated ?

3 (on the right) what connects downregulation of FGFR1 with abnormal DNp63alpha protein stability ?

Besides the confusional scheme layout and symbolism, I also have several doubts about the CONTENT of this scheme.

Some examples:

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2   (on the right) Inactivation of FGF8 in mice causes SHFM ? This is a strong and relatively imprecise statement, considering that SHFM is not a consistent phenotype seen in the absence of FGF8, and in subsequent publications by the same team a proximal-distal instructive role of multiple FGFs has been documented (Mariani et al 2017 doi: 10.1002/dvdy.24480. PMID:28002626; Mariani et al 2008 doi: 10.1038/nature06876. PMID: 18449196)

3   (on the left) Please clarify how downregulation of WNT10B connects with downregulation of DLX5

In my view, the authors should totally re-consider the scheme in Figure 2, deciding to focus on ONE aspect. Such as "documented genetic regulations between SHFM-causing genes" (e.g. transcription factors and target genes). Or, "regulatory protein modification and stability (e.g. p63). Or, "signalling molecules and their receptors". Or, "how SHFM-causing genes could alter AER formation/maintenance/activity".

Another important point is that knowledge on the limb developmental defect at the basis of SHFM has been extensively studied in the mouse model, for obvisous reasons. When trying to bring together data from mouse embryos and data concerning human genetic conditions associated with SHFM, the risk is to authomatically imply that the genetic of the mouse is the same than that of human, which is not entirely true.

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The authors clearly expose and explain the complex pathways that are altered in SHFM malformation. Different genes, belonging to the same molecular cascade, are known to cause SHFM if mutated.

What is missing in this review is the illustration of the work done so far to place the different genes in order in the molecular cascade, that is who is regulating who? Which one is the first actor in the cascade?

The cascade is not fully delineated but the partial data available should be included

Minor points, please pay particular attention to typing errors and capital/ lower case inversion

Date Sent:

09-Jan-2018

Response to the comments of the Reviewers

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RESPONSE: Thank you for these great comments and suggestions. We missed mentioning about BHLHA9. We apologize for this. We added the references and the story about *BHLHA9* and its relationship with SHFM into the manuscript and Figure 2. Yes upregulation of FGF8 may lead to SHFM also. We have used the term "dysregulation" instead of downregulation.

6. The authors should discuss the molecular link between SHFM-related genes and the process of chondrogenesis in limb bud in their discussion on the onset of SHFM phenotype in limb formation. The link of EPS15L1 in chondrogenesis should be considered. EPS15L1 related receptor EGFR and its pathway has important role in chondrogenesis (Yoon et al 2000).

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RESPONSE: We are so sorry. We could not find the relevance of *EPS15L1* gene and SHFM in this paper. From our perspective, the lack of chondrogenesis is due to an insufficient number of mesenchymal cells to allow a digit to form.

7. The authors have focused on the role of FGF8 in SHFM. Accordingly they should discuss its role at cellular level in AER elaborating pathogenesis in the light of available research data. One relevant paper [Gros et al 2010] has discussed effect of the FGF8 on the mobility of cells in the limb bud.

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RESPONSE: Thank you for this valuable comment. We added this into the manuscript as suggested………….. FGF signalling is known to be important in driving cell proliferation, cell survival, and specification of limb mesenchymal cells. It also promotes the velocity of cell movements within the developing limb bud, thereby promoting limb elongation (Gros et al., 2010).[33]

8. The authors should give some details on the molecular cause of intra-familial variability of phenotypic expression of SHFM that would be pretty elaborative for the pathways being discussed.

RESPONSE: This is added in the introduction……………..Intra-familial and inter-individual variability of the SHFM are very high (Fig. 1). Phenotypic variability is most likely the result of genetic background including modifier genes, epigenetic and environmental factors.

9. For common readers, it would be more useful if the authors present clinical features result from mutations in different SHFM genes. In the table mutations reported so far can also be added.

RESPONSE: We tried to find the clinical pictures of SHFM caused by different genes. However, we could not find the clinical pictures of those. The table of mutations of different genes would go beyond the scope of this review. Thank you.

Reviewer: 2

Comments to the Author

The intent of this review is to highlight some key pathways relevant for the comprehension of the congenital limb malformation known as SHFM. The authors combine human genetics with mouse developmental biology to push the concept that FGFs are the key signals that (when perturbed) would cause misorganization of the AER, and that Wnt signaling is upstream of FGFs.

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A. The concepts indicated above are neither wrong nor fully correct, overall the analysis and interpretation of the available data that the author propose appear quite superficial and mis-focused. I did not find this review useful, and on some aspect it is misleading.

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a) Wnt signalling is not ONE signal but rather a wide class of ligands/receptor systems, which are sequence-related but are implicated in nearly all developmental processes, from proliferation to differentiation, from cell polarity to oriented cell division and migration, etc… WHICH of the following the authors propose ? and why ? Wnt10b and Wnt5 have been implicated in SHFM, while Wnt7b is implicated in dorso-ventral orientation, and Wnt3 is implicated in early AER induction. WHICH of these ? Although they are all "WNTs", they activate partially distinct and partially overlapping pathway and certainly carry out distinct functions.

RESPONSE: We agree with this comment. There are several Wnt pathways, but generally no one has been able to assign a specific Wnt to a specific pathway. A single Wnt, such as WNT10B, may be able to activate multiple pathways involved in limb development. The roles of B-catenin-dependent (canonical) signaling in AER formation and limb development are well recognized. But it is possible that other Wnt pathways including non-canonical Wnt signaling pathways may also have roles. (Xi He, person communication). The FGF8-FGF10 regulatory loop play crucial role in limb initiation and AER induction. This loop is controlled by *Wnt2b*, *Wnt3a*, and *Wnt8c* genes through B-catenin-dependent Wnt signaling pathways [Kawakami et al., 2001; MacDonald et al. 2009]. Non-canonical Wnt signaling is also involved in limb development at least in part via *Wnt5a*, a downstream target of Dlx5 which is involved in establishment of the polarization of AER cells of the developing limb. *Wnt5a* is expressed in the limb bud AER and mesenchyme in a distal-high to proximal-low gradient at the same time as *Dlx5*, *Dlx6*, and *p63* [Yamagushi et al., 1999]. Abnormal expression of *Dlx* genes lead to abnormal *Wnt5a* expression in the central AER of the limb bud and subsequent loss of basoapical and planar cell polarity, altered AER organization and function, resulting in SHFM [Conte et al., 2016].

……..We are more specific with Wnt signalling in this resubmission.

b) The same for the FGFs. These are potent mitogenic and patterning signals, but also orient the migration of limb mesenchymal cells. FGF8 appears to play a role in SHFM, however this notion is derived from mouse models. Hence, in the same model, the mouse limb bud, WHAT is the role of (DIRECTLY) altered FGF signaling and AER formation ? Does altered FGF signalling result in altered digit patterning ? This is particularly important, since the rest of the review deals with issues related to digit patterning. The authors should focus on those individual Wnt and FGF ligand/receptor systems directly implicated in SHFM, and examine in depth their individual and combined roles.

RESPONSE:

We see no evidence that altered FGF signaling alters digit patterning, at least what has been reported in the SHFM literature. It is happening too late. Again, during the time period that seems to be critical for SHFM, the migration of mesenchyme cells as reported by Gros et al. is a minor player, at best. We are convinced that digit patterning is not the basis of the defect, but rather, a defective expression of patterning. Our hypothesis is that the defect is due to insufficient mitogenic support for the formation of the central digits due to lack of production of FGF8 by the central AER. There is the difference between initial digital patterning and expression of that pattern.

The issues of digit patterning and proximo-distal extension have been reviewed elsewhere and cannot be illustrated here "generically". **Each signaling molecule DIRECTLY implicated in SHFM should be re-examined in the context of known developmental processes** that take place during limb development. At the end of reading the review the KEY question remains : WHY the central digits are affected (as consequence of the altered Wnt and FGF signaling at the AER) while the other digits are nearly normal ? Which is to say, how can the digit patterning be unaffected while central digits are severely hypoplastic ?

RESPONSE: The remaining digits are not normal. The remaining digits are almost always malformed and syndactylous. That is why we added the proposed diagram of the pathogenesis of syndactyly of the remaining digits (Fig. 3).

B. As correctly stated, SHFM is a limb developmental defect that is closely linked to mis-function of the AER; however there is no clear evidence of an altered digit identity. The fact that the remaining digits appear malformed (hence cannot be assigned digit number based on morphology) is of little meaning. A molecular identity should be assigned and no study that I am aware of has examined this. On the contrary, the expression of SHH, Gremlin and HOX genes is relatively normal in the limbs of mutant mouse models models of SHFM. Therefore, the paragraph "Embryological Mechanisms of Digit Patterning" of this review is scarcely relevant, is poor of scientific content and does not contribute relevant observation or connections.

RESPONSE: Evidently the remaining digits of SHFM appear malformed. We agree that there is no evidence, especially molecular, of altered digit identity, because we have no molecular markers of digit identity. We strongly disagree with the reviewer's assessment of the irrelevance of understanding mechanisms of digital patterning, because what is most critical is understanding the temporal relationship between digit patterning and the actual formation of digits at a later time. There is still disagreement as to how fixed digit patterning is in time.

C. The same can be said about the section "Mirror Image Morphogenesis in SHFM". The section concludes that there is NO mirror image defect in SHFM, something that is expected since the phenotype is linked to altered maintenance and/or activity of the AER which form at a later time with respect to limb induction and general anterior-posterior patterning. *Curiously, the authors state in the Abstract that "mirror image morphogenesis is disrupted in SHFM".* Which one is true ? This appears as an evident contradiction.

RESPONSE: We are sorry. It was a typo. Actually the mirror image morphogenesis is evidently disrupted in SHFM. Previously reported cases in the literature showed asymmetric phenotypes of hands and feet in most if not all cases. That is why we want to bring up the idea of disruption of mirror image morphogenesis. The science behind this concept is lacking but we can only bring up what we see in patients and we are trying to connect what we see with what seen in mouse experiments. We suggest in the

paper that Dlx5 might have an important role in mirror image morphogenesis because most pathways that lead to SHFM are involved *Dlx5* gene and it is interesting to note that olfactory organs of *Dlx5-/-* mice are usually asymmetric.[58] This suggests that *Dlx5* may have important role in mirror image morphogenesis.

D. In the Abstract as well as in the Introduction, the authors mention "mutations of FGF8", however I do not know about these mutations in human, they are not specifically cited in the reference list, and they don't appear in the literature. Please double check this information.
RESPONSE: *Fgf8* mutations in mice is mentioned in the diagram. *FGF8* mutations have not been reported in humans.

E. In the abstract as well as in the Introduction, the authors maintain that since different gene mutations cause an undistinguishable phenotype, this implies that they share a common pathway. This is plausible, but not necessarily true. The indicated genes could cooperate and act in parallel – partially redundant – pathways (known for FGFs and Wnts) or they could act separately and converge onto – and thereby regulate – a common core process, yet to be identified. The authors should be cautious in their statements.
RESPONSE: The intention of our review is to lay down "the principle mechanisms" involved in the pathogenesis of SHFM, unfortunately we have to disregard some detail. The fact is the biology of our body is too complex to include everything in one diagram.

Finally, some citation are wrongly cited. The authors should double check and make sure that each references is correctly placed in the text, and really supports what the text says.
RESPONSE: We have checked and corrected the cited references.

About the scheme proposed in Figure 2
Figure 2 proposes a scheme which I find quite confusing and misleading.

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Another important point is that knowledge on the limb developmental defect at the basis of SHFM has been extensively studied in the mouse model, for obvious reasons. When trying to bring together data from mouse embryos and data concerning human genetic conditions associated with SHFM, **the risk is to automatically imply that the genetic of the mouse is the same than that of human, which is not entirely true.**

RESPONSE: Thank you for these comments. We have made a new diagram and it is much more clear. It illustrates the genes and molecules that involved in SHFM. It summarizes the information we learned from mice and men. We rearranged the diagram and the roles of canonical Wnt signalling and non-canonical Wnt signalling are better illustrated. We agree that this diagram is complicated, so is the biology of our body.

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