IN VIVO SCREEN FOR HEDGEHOG (Hh) PATHWAY ANTAGONISTS

Technical Field

The present invention generally relates to a fish from the family of Cyprinidae comprising an inhibited *Kif7* gene. The present invention also relates to methods of screening for Hedgehog (Hh) pathway antagonists using a fish of the present invention.

Background

Activation of the Hh pathway has been implicated in 25% of all human cancers, making it an important target for the development of anti-cancer therapeutics. To date, all novel anti-Hh therapeutics in clinical trials target the GPCR-like protein, Smoothened (Smo); this is an obligate components of the Hh pathway that acts just downstream of the receptor, Patched (Ptc).

Although some drugs have shown impressive efficacy, there are reports of rapid acquisition of resistance through mutation of Smoothened, underlining the need for the development of drugs against multiple targets in the pathway.

Mutations in Hh pathway genes acting downstream of Smoothened have also been reported to be associated with tumours, emphasizing the need for drugs targeting downstream pathway components.

We have developed a novel in vivo model of Hh pathway activation downstream of Smoothened. The model consists of zebrafish homozygous for loss of function mutations in the Kif7 gene that also carry a GFP-based reporter of Hh

pathway activity. Kif7 acts downstream of Smoothened to negatively regulate the activity of the Gli transcription factors, which mediate the response of cells to Hh signals.

We have discovered that zygotic inactivation of Kif7 in zebrafish is not lethal: however, homozygous mutant animals produce homozygous mutant progeny that show strong upregulation of the Hh GFP reporter gene. As expected, this effect is insensitive to the Smoothened inhibitor, cyclopamine, confirming that the pathway is upregulated downstream of Smoothened.

As the loss of Kif7 derepresses the Hh pathway downstream of Smo, the embryos provide the basis for a screen for a highly specific class of Hh inhibitors. Kif7 mutant mouse embryonic fibrobalsts (MEFs) have previously been reported but their use in screening for specific inhibitors has not been proposed. Advantageously, the screening method of the present invention is robust, non-quantitative, cheap and based on an in vivo activity assay.

Summary

According to a first aspect, there is provided a fish from the family of Cyprinidae comprising an inhibited *Kif7* gene and wherein the fish is homozygous for the inhibited *Kif7* gene.

In a second aspect, there is provided a zebrafish comprising an inactivated *Kif7* gene and a reporter gene.

In a third aspect, there is provided a *Danio rerio* comprising two inactivated copies of the *Kif7* gene and a

reporter gene, wherein the *Kif7* gene is inactivated using zinc finger nuclease-induced mutagenesis and the reporter gene encodes green fluorescent protein.

In a fourth aspect, there is provided a method of identifying a compound capable of inhibiting the Hedgehog cell signalling pathway comprising the following steps:

(a) contacting the compound with a fish of any one of the preceding claims and measuring the expression level of the reporter gene;

(b) contacting a control compound with a control group of the fish and measuring the expression level of the reporter gene;

(c) comparing the reporter gene expression levels obtained from steps (a) and (b);

wherein a decreased expression of the report gene relative to the control group indicated that the compound is capable of inhibiting the Hedgehog cell signalling pathway.

Brief Description of Drawings

The accompanying drawings illustrate a disclosed embodiment and serves to explain the principles of the disclosed embodiment. It is to be understood, however, that the drawings are designed for purposes of illustration only, and not as a definition of the limits of the invention.

Figure 1 is a schematic diagram showing what happens when there is no Hh binding to the Ptc receptor of a normal cell. This results in Ptc repressing the activity of smoothened and thereby the activation of the downstream signaling pathway. In the absence of Smo activity, Kif7

inhibits Gli transcriptional activity and hence there is no or reduced expression of the reporter protein.

Figure 2 is a schematic diagram showing what happens when Hh binds to the Ptc receptor in a normal cell. When Hh binds to Ptc, this removes the inhibitory effect of Ptc on Smo, which in turn results in Smo inhibiting the activity of Kif7. This allows Gli to activate the *Hh* reporter gene resulting in the expression of the reporter protein.

Figure 3 is a schematic diagram showing what happens when a normal cell is exposed to Hh and an inhibitor of Smo, such as Cyclopamine. Binding of Cyclopamine to Smo results in inactivation of the latter, even in the absence of Ptc mediated inhibition; this in turn removes the inhibitory effect of Smo upon Kif7. This allows Kif7 to inhibit the activity of Gli, resulting in no or reduced expression of the reporter protein.

Figure 4 is a schematic diagram showing what happens in a Kif7 homozygous mutant cell. Since Kif7 is inactivated, the activity of Gli is not repressed and this results in high expression levels of the reporter protein.

Figure 5 is a schematic diagram showing what happens in a Kif7 homozygous mutant cell when the cell is exposed to Cyclopamine. The binding of Cyclopamine to Smo results in the removal of the inhibitory effect of Smo upon Kif7. However, in contrast to a normal cell (see Figure 3), because Kif7 is inactivated, Gli activity is not affected

and hence the reporter protein is expressed at high levels.

Figure 6 is a schematic diagram showing what happens when a Kif7 homozygous mutant cell is exposed to a novel chemical inhibitor which affects the activity of Gli, or of factors required for its activation, stability or nuclear import. In this case, although Kif7 is inactivated and therefore unable to regulate the activity of Gli, because the novel inhibitor acts directly upon Gli or positive modulators of its activity, this results in no or reduced expression of the reporter protein.

Examples

Non-limiting examples of the invention will be further described in greater detail by reference to specific Examples, which should not be construed as in any way limiting the scope of the invention.

Example 1 - Generation, selection and genotyping of kif7 mutant alleles

Plasmids encoding Zinc-finger nucleases (ZFN) specific for the Zebrafish *kif7* (set 3) gene were purchased from Sigma. The Zinc Finger Nuclease Binding Site is shown below (ZF-nucleotide recognition site in bold italics).

Set 3:
24631 tcATCCAAATTCCATTTTGTGGAC***CTGGCAGGATCAGAG***cg
24630 ag***TAGGTTTAAGGTAAAA***CACCTGGACCGTCCTAGTCTCgc

Capped polyadenylated RNA from each plasmid was produced by *in vitro* transcription and a range of doses was injected into one-cell stage zebrafish embryos. Embryos were injected with approximately 100pg had around 30% rate of deformity at 24 hpf (hours post-fertilisation). Genomic

DNA prepared from non-deformed 24hpf embryos injected with this dose was used as a PCR template to analyse potential somatic mutations.

Roche Titanium 454 amplicon sequencing showed that 6.3% of the amplicon molecules had insertions or deletions at the target site. G0 adults derived from embryos injected with ZFN capped RNA were in-crossed and their progenies (G1) individually genotyped by PCR using the forward primer (Kif7 exF2:CGAGGTGCTGAGTCTCTTAGAGT) and reverse primer (Kif7 ex R1:TGAATCCCTGTATGGGATATGGGT) followed by Sanger sequencing.

Founders transmitting two different alleles (kif7Δ8, an 8bp del: GGAACCTGG) and kif7Δ7, a 7bp del: TTGTGGA) were selected and used to establish stable mutant lines. The full nucleic acid sequence of each allele is shown below.

Underline + Highlighting = kif7-delta8 mutant
Bold italics + Underline = kif7-delta7 mutant

>zf-kif7-cDNA (mutants marked)
ATGTCTCCAAAAGGAGTCGGCCACAGTAAGGTGGAAGAAAGTGCTGTACAGGTGGCCGTCCGCGTGCGTC
CGTTGCTTCCCAAAGAGATTTTGCACAGCCATGAGAGCTGCATCACTTCAGACCCAGAGGAGCGGAGGGT
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Delta-8 protein (8 aa after mutation)

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R V R P L L P K E I L H S H E S C I T S
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D P E E R R V T L G N D R H F H C D F V
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F E D G S T Q E E V Y T N C V Q P L I E
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A F F H G F N A T V S A Y G Q T G S G K
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T Y T I G E A S I S A F R D D E Q G I I
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ca
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Kif7 wt protein seq

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```

Applications

The present invention may be used to screen for novel inhibitors of the Hh pathway acting downstream of Smoothened.

Advantageously, the method of screening is robust, non-quantitative, cheap and based on an in vivo assay.

Advantageously, a large number of homozygous Kif7 mutant fish embryos can be easily produced, allowing very rapid visual screening of thousands of compounds in a matter of weeks.

It will be apparent that various other modifications and adaptations of the invention will be apparent to the person skilled in the art after reading the foregoing disclosure without departing from the spirit and scope of the invention and it is intended that all such modifications and adaptations come within the scope of the appended claims.

Claims

1. A fish from the family of Cyprinidae comprising an inhibited *Kif7* gene and wherein the fish is homozygous for the inhibited *Kif7* gene.

2. The fish of claim 1, further comprising a reporter gene.

3. The fish of claim 1 or 2, wherein the fish is of the Danioninae sub-family.

4. The fish of claim 3, wherein the fish is of the Danio or Devario genus or which is selected from any one of the following species: *Danio rerio (Zebrafish)*, *Danio aesculapii*, *Danio albolineatus*, *Danio choprae*, *Danio dangila*, *Danio erythromicron*, *Danio feegradei*, *Danio jaintianensis*, *Danio kerri*, *Danio kyathit*, *Danio margaritatus*, *Danio meghalayensis*, *Danio muongthanhensis*, *Danio nigrofasciatus*, *Danio quagga*, *Danio quangbinhensis*, *Danio roseus*, *Danio tinwini* and *Danio trangi*.

5. A zebrafish comprising an inactivated *Kif7* gene and a reporter gene.

6. The fish of any one of the preceding claims, wherein the fish is an embryo, or a larva or adult.

7. The fish of any of the preceding claims wherein the *Kif7* gene is inhibited completely or partially.

8. The fish of claim 7, wherein the *Kif7* gene is inhibited temporarily or permanently.

9. The fish of claim 8, wherein the *Kif7* gene is inhibited temporarily using one or more of the following: short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), short hairpin RNA (shRNA) and morpholinio oligonucleotides.

10. The fish of claim 8 wherein the *Kif7* gene is inactivated permanently by introducing one or more mutations into the *Kif7* gene or any sequence controlling expression of the Kif7 gene which results in a loss of function or lack of expression of the *Kif7* gene.

11. The fish of claim 10, wherein the mutations are introduced by one or more of the following methods: site-directed mutagenesis, insertional mutagenesis, PCR site-directed mutagenesis, plasmid mutagenesis, cassette mutagenesis and zinc finger nuclease-induced mutagenesis.

12. The fish of claim 11, wherein both copies of the *Kif7* gene are inactivated.

13. The fish of any of claims 2 to 12, wherein the reporter gene encodes a visually identifiable protein.

14. The fish of claim 13, wherein the protein is a fluorescent protein, luminescent protein, or beta-galactosidase.

15. The fish of claim 14, wherein the fluorescent protein is green fluorescent protein, red fluorescent protein or reef coral fluorescent protein.

16. The fish of any one of claims 2 to 15, wherein the reporter gene is operatively linked to a regulatory region of one or more genes of the Hedgehog pathway.

17. The fish of claim 16, wherein the gene of the Hedgehog pathway is the *Eng2a* gene and/or the *slow myosin heavy chain 1* gene.

18. The fish of claim 16 or 17, wherein the regulatory region is a cis-regulatory region.

19. A *Danio rerio* comprising two inactivated copies of the *Kif7* gene and a reporter gene, wherein the *Kif7* gene is inactivated using zinc finger nuclease-induced mutagenesis and the reporter gene encodes green fluorescent protein.

20. A method of identifying a compound capable of inhibiting the Hedgehog cell signalling pathway comprising the following steps:

(a) contacting the compound with a fish of any one of the preceding claims and measuring the expression level of the reporter gene;

(b) contacting a control compound with a control group of the fish and measuring the expression level of the reporter gene;

(c) comparing the reporter gene expression levels obtained from steps (a) and (b);

wherein a decreased expression of the report gene relative to the control group indicates that the compound is capable of inhibiting the Hedgehog cell signalling pathway.

21. The method of claim 20, wherein the fish is contacted with the compound by injecting the compound into the fish.

22. The method of claim 21, wherein the compound is injected into the fish in conjunction with a carrier.

23. The method of claim 22, wherein the carrier is a solvent, lipid or peptide.

24. The method of any one of claims 20 to 23, wherein the method refers to identifying a compound capable of inhibiting the Hedgehog cell signaling pathway downstream of Kif7 protein.

25. The method of any one of claims 20 to 24, wherein the expression level of the reporter gene is measured by light or fluorescent microscopy.