Table 1
Characterization of somitic genes obtained using the subtractive strategy

Clone no.	Character	EP in WT	EP in Mesp2$^{-/-}$
ST347	Hes7	PSM (oscillates)	No change
ST371	laminin alpha 1	PSM (strong at S-1)	Expands anteriorly
ST556	Glcci1*	AER, somite, NT (dorsal)	No change
ST623	Laminin 5	PSM-1, somite anterior	Disappears
ST676 ST686 ST762 PTS286 PTS360	LEF1	PSM (strong at S-1)	Expands anteriorly (S-1 region)
ST313	Neuropilin-2	Rostral compartment of somites	Disappears
ST736	Axin2	PSM (oscillates) (S-1 region)	Expands anteriorly (S-1 region)
PTS124 PTS338	SemaF-Cap3	PSM, somite	Downregulated
PTS149	DOC4/Nrg1	PSM, somite, head	No change
PTS179	Slit3	Tailbud, somite, DM	No change
PTS250 PTS426 PTS607	Hoxc13	Tailbud	No change
PTS363	Tropomyosin	Tailbud	No change
PTS378#	No gene prediction	PSM(S-1)	Upregulated
PTS474	ETV5	Tailbud, PSM(S0)	Downregulated (S0 region)
PTS485	MOCH**	somite posterior	No change
PTS542	Nkd2	PSM, somite (dorsal)	No change
PTS635	Calsyntenin-2	PSM(S-1), somite	No change
PTS646	Ets2	PSM (strong at S-1)	Downregulated

EP, expression pattern; WT, wild-type, PSM, presomitic mesoderm; AER, apical ectodermal ridge; NT, neural tube; DM, dermomyotome; *, glucocorticoid induced transcript1; **, mitochondrial oxodicarboxylate carrier homolog; #, GenBank accession number (AB178168).

2. Results

2.1. Screening strategy

Our study has two fundamental aims; to isolate and identify novel genes that are expressed specifically in the PSM and to characterize novel genes that function downstream of Mesp2. To accomplish these goals, we constructed two subtractive cDNA libraries, designated PTS and ST (Fig. 1). The enrichment of specific genes was validated by PCR, using either *G3PDH*- (a ubiquitously expressed gene) or *Mesp2*- (a PSM specific gene) specific primers. *G3PDH* cDNA was found to have been successfully depleted, whereas *Mesp2* cDNA was still evident after repeated subtractions (Fig. 1), suggesting that the subtractive hybridizations had worked effectively. After partial sequencing of randomly selected clones (406 for the PTS and 636 for the ST libraries), an initial search of the GenBank database in NCBI was done using blastn and known genes were removed. As a further screening strategy, we utilized the whole mount ISH technique, using the tail regions of 11.5 dpc embryos. The total number of clones that we subsequently screened by ISH were 251 and 280, for PTS and ST libraries, respectively.

After completion of the ISH screening, we identified more than 30 clones that showed expression in the somite and/or PSM regions. Further sequencing and homology searches using the Celera database identified some of these as regions of known genes (Table 1).

2.2. Characterization of genes obtained by subtractive hybridization

Blastn searches of the GenBank and Celera genome databases identified genes, such as *lymphoid enhancer factor-1* (*LEF1*), that were represented by a number of redundant clones. The clones ST676, PTS566, and PTS629 encode portions of *LEF1* cDNA and ST686, ST762, PTS286, and PTS360 were also identified as intronic sequences of the *LEF1* gene (Table 1 and Fig. 2). During this screening we often obtained intronic DNAs, probably due to the unspliced heterogeneous RNAs since we prepared RNA from whole cells containing nuclei. The intronic DNA fragments sometimes gave rise to different expression patterns, compared with those obtained using exon probes, and these were initially classified differently, as in the case of *LEF1*(Fig. 2) a known nuclear effecter of the Wnt/β-catenin signaling pathway (Porfiri et al., 1997; Hsu et al., 1998). The expression of intronic *LEF1* was observed as an intense band in either S-1 or S-2 of the PSM, in a pattern similar to *Mesp2*, and a lower intensity signal was also observed in the middle PSM. Interestingly the expression was expanded anteriorly in *Mesp2*-null embryos, which is also observed for several genes such as *L-fng* or *Dll1* (Nomura-Kitabayashi et al., 2002). The frequent appearance of this gene, showing these expression patterns, indicated that our strategy could successfully isolate genes that are specifically expressed in the PSM. This was also confirmed for the ST library, from which we isolated two clones, ST313 and ST623, which were identified as *neuropilin-2* and *laminin5*, respectively, and these expression profiles were observed to be confined to the rostral part of somites and are downregulated in Mesp2-null embryos (Table 1 and Fig. 2). Another ST clone, ST371 was identified to be an intronic sequence for *laminin alpha1*, which expression was expanded anteriorly in Mesp2-null embryos (Table 1 and Fig. 2).

Homology searches of the Celera database also determined that clone ST736 contained sequences encoding Axin2 (also named conductin) which functions as a negative regulator of Wnt signaling by inducing β-catenin degradation (Behrens et al., 1998). The expression pattern of

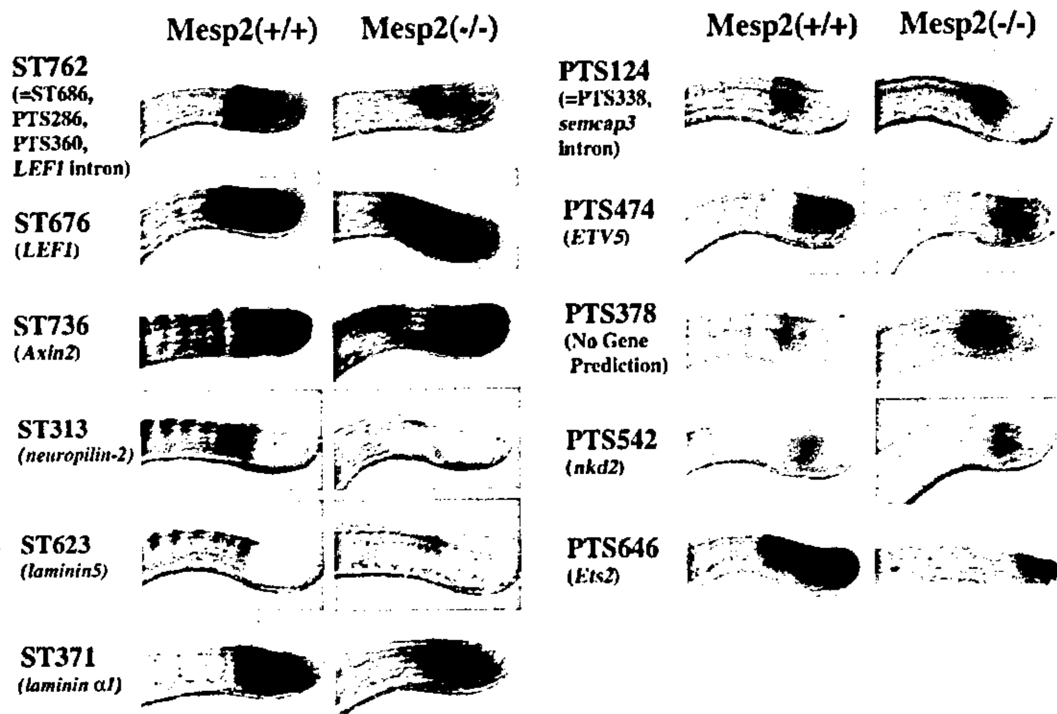

Fig. 2. Expression patterns of cDNA clones selected by ISH screening. ISH were performed with the tail region of wild-type embryos at 11.5 dpc (left side), in comparison to Mesp2-null mutants (right side).

mouse *Axin2* exhibits oscillation in the PSM (Aulehla et al., 2003), with different phase properties from those of *L-fng*, which is an oscillating gene in the Notch signaling pathway. Interestingly, although *Axin2* expression still oscillates when Notch signaling is impaired, a stable stripe of *Axin2* transcripts, in the anterior-most PSM of wild-type embryos, was not observed in Dll1-null mutants (Aulehla et al., 2003). We isolated this clone from our ST library, which may indicate that *Axin2* expression is controlled either directly or indirectly by Mesp2. As shown in Fig. 2, we also observed that the anterior stripe of the *Axin2* transcripts became diffuse and did not form a clear band in Mesp2-null embryos. Celera database searches indicated that the clones PTS124 and PTS338 were intronic sequences of '*M-SEM F cytoplasmic domain-associated protein 3*' (*semcap3*), the expression of which becomes diffuse in the Mesp2-null embryo (Fig. 2). A *Xenopus* homolog of the semcap/GIPC family, kermit, interacts directly with the cytoplasmic portion of the Wnt receptor, frizzled, via its PDZ domain and modulates its signaling activity (Rasmussen et al., 2001; Tan et al., 2001). However, semcap3 has low homology with other family members, and no function has so far been postulated for this protein. PTS474 was identified as '*Ets variant gene 5 (ETV5)*', encoding a transcription factor that contains an Ets DNA binding domain. *ETV5* is expressed in both the tailbud and middle PSM, but not in the anterior-most PSM, and this expression subsequently reappears at the segmental border (Fig. 2). In the Mesp2-null embryo, however, the anterior expression of *ETV5* becomes indistinct. Recently, it has been reported that mouse *ETV5* is expressed in the epithelium of the developing lung and plays a role in epithelial–mesenchymal interactions during lung organogenesis (Liu et al., 2003), but its function in the PSM is still unknown. PTS642 was identified to encode Ets2, another Ets-transcription factor and the expression is downregulated in Mesp2-null embryo (Fig. 2). The expression pattern during morphogenesis has been described (Ristevski et al., 2002). Targeted mutation of Ets2 is reported to result in embryonic lethal before 8.5 dpc due to the defective trophoblast function. However, rescued mice by aggregation with tetraploid embryos are viable and no defect in somitogenesis is reported (Yamamoto et al., 1998). Using either Celera or GenBank database searches, there was no homology detected between PTS378 and known genes. In addition, this clone is unique as it is up-regulated in the absence of Mesp2 (Fig. 2), which was a rare finding during our subtraction analyses. We speculate that this clone may in fact be a member of the non-coding RNAs.

PTS542 was identified as a gene encoding Naked cuticle 2 (Nkd2), the transcript of which is detected in the middle PSM, and it seems that Mesp2 mutation does not affect the expression pattern of *nkd2*. *Naked cuticle* was first identified as a *Drosophila* segment polarity gene (Zeng et al., 2000) and mutation of this gene in the fly embryo causes similar phenotypes to excess Wg expression, indicating that Naked cuticle functions as an inhibitor of Wg signaling.

Since another Nkd protein in the mouse, Nkd1, can antagonize Wnt signaling in both cultured cells and in *Drosophila* embryos (Wharton et al., 2001), we determined whether *nkd1* is also expressed in the PSM and found that it exhibited an interesting expression pattern.

2.3. Mouse nkd1 mRNA oscillates in the PSM

nkd1 mRNA is first detected in the PSM and in the central nervous system of the 8.0 dpc embryo (Fig. 3A,B). In the 10.5 dpc embryo, *nkd1* expression is then observed in newly formed somites and in the neural tube, and was found to be maintained in the PSM (Fig. 3C). However, this expression pattern in the PSM varied among embryos and the signal was detected in either the middle of the PSM and tailbud (Fig. 3D) or in the more anterior PSM without tailbud (Fig. 3E). This suggests that the *nkd1* expression pattern changes in a short time in the PSM. To further analyze the precise transcriptional regulation of *nkd1*, we designed an RNA probe that was complementary to intronic sequences within the *nkd1* gene. Because this probe does not hybridize to mature spliced mRNA, we could therefore exclusively detect premature *nkd1* mRNA. Using the intronic RNA probe, we detected various expression patterns of *nkd1* in 11.5 dpc embryos (Fig. 3F–H), which could be categorized into three different phases, similar to other cyclic genes (Palmeirim et al., 1997; Bessho et al., 2001; Aulehla et al., 2003): Phase I: lower *nkd1* signal intensities in the tailbud. Phase II: *nkd1* transcripts are evident in the middle of the PSM in addition to the tailbud. Phase III: the *nkd1* signals shift more anteriorly. These results indicate that *nkd1* expression oscillates in the PSM during somitogenesis. However, another member of this family of genes, *nkd2* did not exhibit such periodic expression, although its expression was observed in the PSM (Fig. 2).

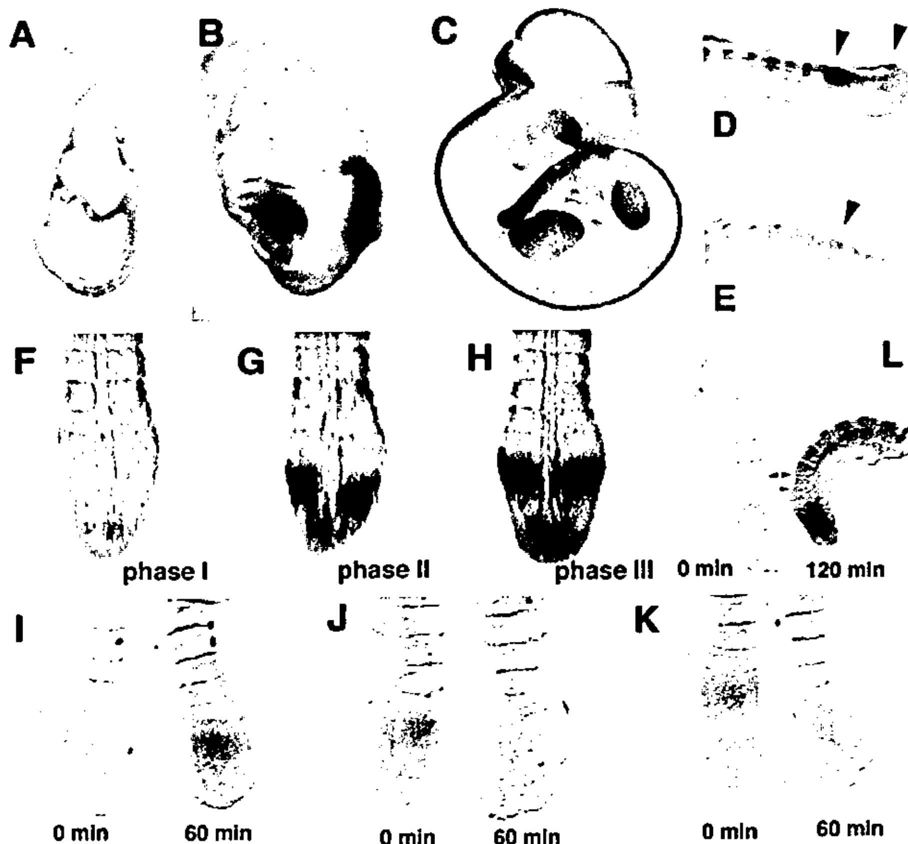

Fig. 3. Expression patterns of mouse *nkd1*. *nkd1* mRNA was detected with an exon probe at 7.5 dpc (A), 8.5 dpc (B), 10.5 dpc (C–E). *nkd1* transcripts are first detected in the central nervous system and in the PSM of 8.5 dpc embryos (B). In the 10.5 dpc embryo, *nkd1* expression is detected in the neural tube, dermomyotomes, somites and the PSM (C). Different expression patterns were detected using the exon probe (D,E). Arrowheads indicate signals in the PSM. (F–H) *nkd1* transcripts were detected using an intron probe in the tail region of 11.5 dpc embryos. Note that the expression is very weak in phase I (F), becomes stronger in the middle PSM in phase II (G) and is confined to the anterior PSM in phase III (H). (I–M) An embryo half culture experiment indicating the oscillation of *nkd1* expression. The 11.5 dpc embryo tails were cut into two halves at the midline. The left side was fixed immediately and the right side was fixed after 60 min (I–K) or 120 min (L) cultivation. Black and red arrowheads in (L) indicate segment border existed at 0 min and a newly formed border after 120 min, respectively. An intron probe was used for the ISH.

To further confirm the cyclic expression profile of *nkd1*, we performed in vitro cultures of bisected PSM, by fixing one half of the isolated tissue immediately and the additional half after a period of culturing. After 60 min, the expression patterns of *nkd1* in cultured PSM cells were altered from those of the uncultured portion (Fig. 3I–K). The change was most evident in the middle to anterior PSM, where the expression was seen to go from zero to strong levels and vice versa. After 120 min, the same pattern was observed which accompanied the formation of a new somite (Fig. 3L). These observations demonstrate that *nkd1* expression oscillates in the PSM during somitogenesis.

2.4. Comparisons of nkd1 expression patterns with other oscillatory genes

There are several genes that show periodic expression patterns during somitogenesis. The Notch-signaling related genes, *L-fng* and *hes7*, are transcribed with a similar oscillatory phase (Bessho et al., 2001), whereas the Wnt inhibitor *Axin2* has a different expression phase from these factors (Aulehla et al., 2003). To elucidate which of these signaling pathways is involved in the regulation of *nkd1* expression, we compared the expression patterns of *nkd1* with those of either *L-fng* or *Axin2* in dissected embryo halves. Surprisingly, *nkd1* was found to be expressed in the same phase as *L-fng* in the middle PSM (Fig. 4A–C), although its expression was not observed as a narrow band in the anterior PSM, as is the case for *L-fng* (Fig. 4B). These expression patterns are similar to those of the *hes7* transcripts detected with an intronic probe (Bessho et al., 2003). The oscillatory expression of both *L-fng* and of *hes7* itself is negatively controlled by Hes7 (Bessho et al., 2003). Thus, it appeared possible that *nkd1* expression would also be controlled by Hes7. On the other hand, the expression domains of *nkd1* and *Axin2* were obviously out of phase (Fig. 4D–F). For example, *Axin2* expression is not highly upregulated in the middle PSM where *nkd1* is strongly detected as phase II (Fig. 4E). *nkd1* expression is also detected as phase III in regions where *Axin2* expression is downregulated (Fig. 4F) and these spatial relationships resemble those of *Axin2* versus *L-fng* (Aulehla et al., 2003). These observations suggest that the oscillation of *nkd1* expression is regulated in a similar fashion to *L-fng*.

2.5. The oscillation of nkd1 expression is controlled by Hes7

To further confirm that the regulation of *nkd1* is under the control of the segmentation clock, via Notch signaling, we examined *nkd1* expression in the absence of Hes7 (Fig. 5A–C). In each of the Hes7-null embryos that we analyzed ($n=7$), the pattern of *nkd1* mRNA was similar. Strong expression was observed at the middle PSM, and weaker signals were observed in the remaining PSM. In comparison to wild-type embryos (Fig. 5D,E), it appeared that *nkd1* expression levels are upregulated and that its oscillatory patterns are arrested in Hes7 null-mice.

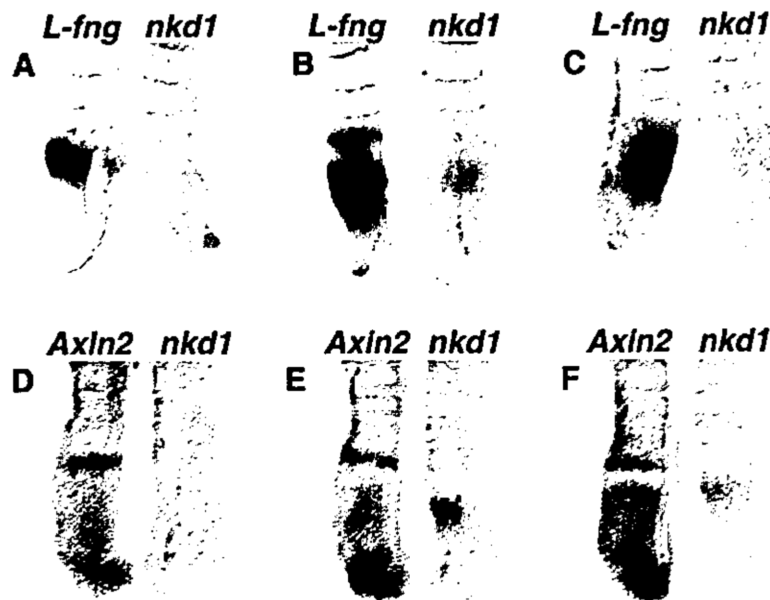

Fig. 4. Comparisons of the *nkd1* expression domains with the regions showing expression of other cyclic genes. (A–C) 11.5 dpc tail halves were stained with an *L-fng* probe (left) or an *nkd1* intron probe (right). The major expression domains overlapped with the exception of the anterior-most band of *L-fng* in B. (D–F) Embryo halves were stained with *Axin2* probe (left) or *nkd1* intron probe (right). The oscillatory phases of the expression patterns of *Axin2* and *nkd1* were found not to be synchronized.

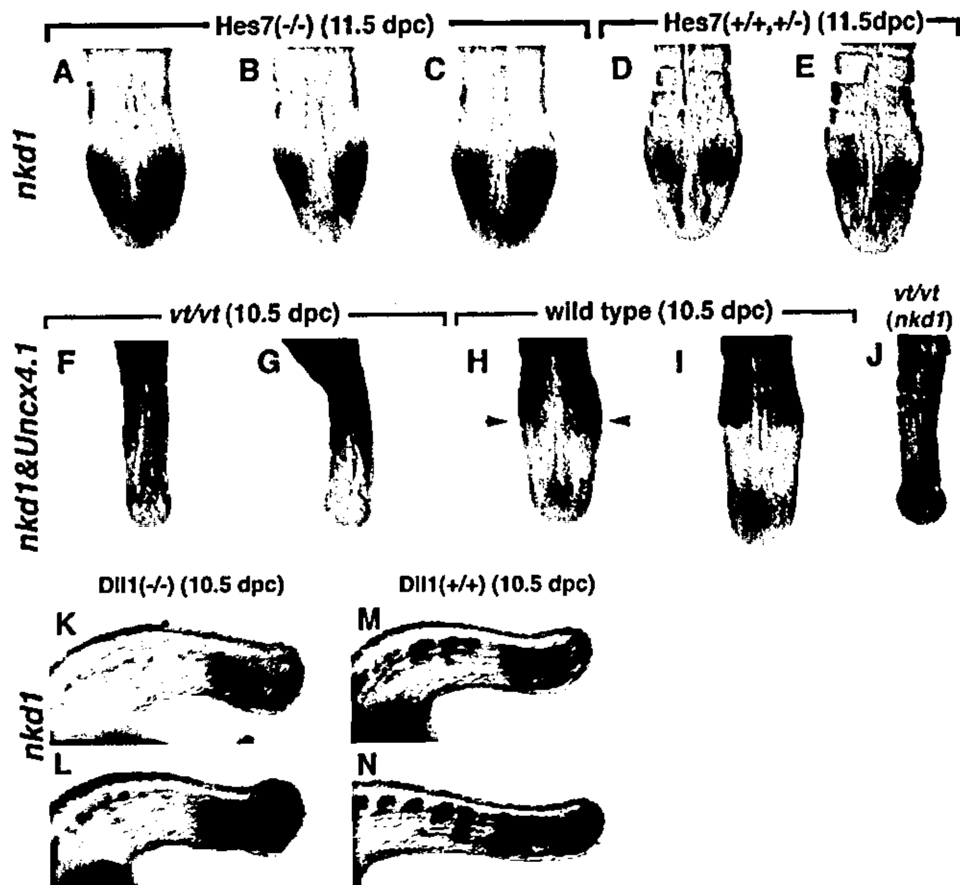

Fig. 5. *nkd1* oscillation is arrested in Hes7-null mice and its expression decreases in *vt/vt* mice but not in Dll1-null mice. (A–E) *nkd1* transcription was detected with an intron probe in either 11.5 dpc Hes7-null (A–C) or wild-type (D–E) littermate embryos. (F–I) *nkd1* expression in *vt/vt* and wild-type embryos was detected using an intron probe. *Uncx4.1* was used as an internal control. (J) *nkd1* probe alone was used in the *vt/vt* embryo. In the PSM of *vt/vt* embryos, *nkd1* mRNA is downregulated. The expression observed in the anterior PSM of wild-type embryo (arrowhead) corresponds to that of *nkd1* since *Uncx4.1* is expressed in the caudal portion of segmented somites. (K–N) *nkd1* expression in Dll1-null and wild-type embryos showing unaltered levels.

This suggests that the cyclic expression of *nkd1* depends on the negative regulation, either directly or indirectly, by the Hes7 transcription factor.

2.6. *nkd1 expression may be induced by Wnt signaling*

In fly embryos lacking *Wg*, *nkd* transcription initiates normally but is not subsequently maintained (Zeng et al., 2000). In human colon tumors, initiated by activated Wnt/β-catenin signaling, *hnkd* (*NKD1*) expression is elevated and experimental reduction of β-catenin leads to a decrease in *nkd1* mRNA (Yan et al., 2001b). These findings suggest that *nkd1* expression and/or maintenance depends on Wnt signaling. To determine whether Wnt signals affect *nkd1* expression during somitogenesis, we investigated *nkd1* expression in the *Wnt3a* hypomorphic mutant *vestigial tail* (*vt*) embryo. The *Wnt3a* coding sequence in the *vt* mouse is intact but its expression is markedly reduced in the tailbud from 9.5 dpc because of the mutation in the *Wnt3a* regulatory region (Greco et al., 1996). In *vt/vt* embryos ($n=4$), *nkd1* transcription is greatly reduced in the middle PSM and tailbud regions (Fig. 5F,G and data not shown) whereas expression levels in the neural tube and newly formed somites are normal (Fig. 5J). The expression levels of *L-fng*, however, are not altered in *vt/vt* embryos (Aulehla et al., 2003), although no oscillation is observed in these mutants. In the PSM, we have shown that *nkd1* mRNA oscillations are produced by a similar mechanism to *L-fng*, via negative regulation by Hes7. However, the induction and/or maintenance of *nkd1* transcription may require a different signal from *L-fng*, and we speculate that this is probably provided via Wnt3a. To further confirm this possibility, we have analyzed *nkd1* expression in Dll1-null embryos. As shown in (Fig. 5K–N), no reduction was observed, emphasizing that *nkd1* expression itself is independent of Notch signaling. However, no clear oscillation was observed, which also suggests Notch-signal dependent oscillation of *nkd1*.

Taken together, our data suggest that a possible crosstalk between the Notch and Wnt signaling mechanisms and that

their reciprocal regulation might therefore be important for generating the cyclic waves required for refining somitogenesis.

3. Discussion

We have employed a cDNA subtraction method to isolate previously uncharacterized genes that either function downstream of Mesp2 and/or are specifically expressed in the PSM. We successfully isolated several genes that are putatively involved in somitogenesis, and among these we identified several Wnt-signaling related factors. The most abundant gene identified in our screen was *LEF1*, the expression of which is widely observed in the PSM but is detected at its strongest levels in the anterior PSM just prior to segment border formation. *nkd2* is transcribed only in the middle PSM but we have demonstrated that *nkd1* expression oscillates in the PSM during somitogenesis. These facts may indicate that Wnt signaling has an important role in somitogenesis, consistent with previous findings (Aulehla et al., 2003).

It is generally believed that the spatial reiteration of somites is based on the periodicity generated by the molecular segmentation clock. Several Notch-signaling related factors are known components of the molecular clock during somitogenesis (Pourquié, 2001). Additionally, mice lacking Notch-related factors fail to form regular somites, providing strong evidence that the molecular clock organized by Notch signaling is required for formation of the reiterated somite structure. One of the best characterized molecular clock components is Hes7 (Bessho et al., 2003), the upregulation of which depends on Notch signaling. Both the auto-inhibitory activity and instability of the Hes7 protein are responsible for its oscillatory expression properties. Recently, it has been reported that *Axin2* expression also oscillates and that Wnt3a is required for the oscillation of Notch signaling in the PSM, which lead to a postulated link between Wnt/β-catenin signaling and the segmentation clock (Aulehla et al., 2003). However, the mechanisms underlying the regulation of these oscillations have not yet been determined.

We have found that *nkd1* expression exhibits oscillation with a similar phase to *L-fng* during somitogenesis, and that this oscillation is arrested and the expression is upregulated in Hes7-null embryos. These observations strongly suggest that *nkd1* transcription is suppressed by Hes7, with concomitant generation of its cyclic expression. To determine whether Hes7 can bind to the *nkd1* enhancer directly, we attempted to find a Hes7 binding site upstream of the *nkd1* gene. However, some of the 5′ flanking sequences of *nkd1* are absent from the databases and we have not yet fully sequenced the genomic region and so far not found any E-box or N-box sites within the available sequences. Future enhancer analysis of *nkd1* will therefore be necessary to properly determine this possibility.

In the embryo of the Wnt3a hypomorphic mutant, *vestigial tail*, the expression of *nkd1* is greatly reduced in the PSM. This suggests the possibility that Wnt signaling (mediated by Wnt3a) regulates the expression of *nkd1* in the PSM. This would be consistent with previous reports showing that the maintenance of *Drosophila nkd* expression depends on Wg function (Zeng et al., 2000) and that activated Wnt signals induce elevated *hnkd* expression levels in human colon tumors (Yan et al., 2001b). However, as the level of *nkd1* expression is increased and the oscillation of *nkd1* is disrupted in the Hes7-null mutant, this indicates that *nkd1* is under the control of Notch signaling as mentioned above. Since Notch signaling is arrested in the absence of Wnt signaling, it was feasible that *nkd1* oscillation would be arrested in *vt/vt* embryos. It is also possible that the reduction in *nkd1* expression in *vt/vt* embryos is a direct effect of the lack of Wnt signaling or an indirect effect of arrested Notch signaling. However, we think the latter is unlikely since the level of *nkd1* expression is unaltered in Dll1-null embryos. In the neural tube or in young somites, *nkd1* is expressed even in the *vt/vt* mutant, which indicates that additional Wnt signals or possibly a different pathway may regulate *nkd1* transcription in these regions.

The comparison between the expression domains of different cyclic genes is summarized in Fig. 6A. The *nkd1* expression pattern is similar to that of both *hes7* and *L-fng*, but is different from *Axin2*. The reason why *Axin2* and *nkd1* exhibit different expression patterns, irrespective of the fact that both appear to be induced by Wnt signaling, is currently unknown. One possibility is that *nkd1*, but not *Axin2*, oscillation is regulated by Notch signaling, which results in synchronization of *nkd1* oscillation with Notch signaling. In other words, the initial induction of *nkd1* might be induced by Wnt signaling but its regulation is under the control of Notch signaling. However, the expression patterns of Notch-related genes and *Axin2* are not completely mutually exclusive. They appear to be alternate in the tailbud region but they do align in the anterior PSM where *Mesp2* is expressed. Wnt3a appears to induce the expression of *Axin2* directly, but *Wnt3a* mRNA is detected only in the tailbud (Aulehla et al., 2003). Considering that Axin2 is expressed as a detectable band in the anterior PSM and that its expression is altered in Mesp2-null embryos, the expression pattern of *Axin2* may be regulated by Mesp2 in the anterior PSM. The direct targets of Mesp2 have not yet been characterized but many genes that are expressed in the anterior PSM are affected (downregulated or anteriorly expanded) by Mesp2, including *Notch1*, *Notch2*, *Fgfr1*, *Dll1*, *EphA4*, *Papc*, *Cer1* and *L-fng* (Saga et al., 1997; Takahashi et al., 2000; Nomura-Kitabayashi et al., 2002). Among them, EphA4 is implicated in segment border formation and ectodermal signaling is also required for *EphA4* induction (Schmidt et al., 2001), which may suggest that downstream genes of Wnt signal from the ectoderm might interact with Mesp2 to induce *EphA4*. *nkd1* is not

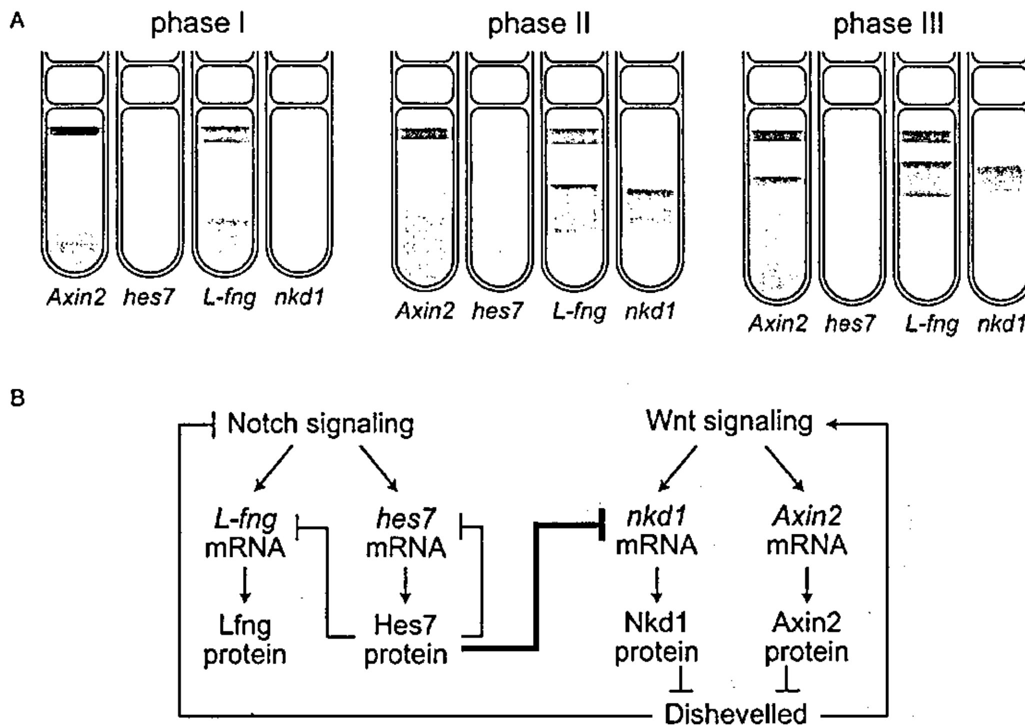

Fig. 6. (A) A schematic diagram of the expression patterns of oscillatory genes in the PSM of 11.5 dpc embryos. PhaseI: *Axin2*, *hes7* and *L-fng* show similar expression patterns in the tailbud and anterior PSM. *nkd1* transcription is greatly downregulated in this phase. PhaseII: The expression domain of *Axin2* is more extensive in the tailbud region. *hes7*, *L-fng* and *nkd1* are all expressed in the same region of the middle PSM, whereas *nkd1* is not detected in the anterior PSM in a band-like pattern. PhaseIII: *Axin2* expression is downregulated in the middle PSM. Around S-1, the expression of all four genes overlaps. (B) Possible interactions between Notch and Wnt signaling. Our findings are integrated into the previously proposed schemes of Bessho et al. (2003) and Aulehla et al. (2003). We propose that Wnt signaling may be regulated by Notch signals via the transcriptional regulation of *nkd1* by Hes7. It has been reported previously that Wnt signaling may regulate Notch signaling via NICD inhibition by Dishevelled (Axelrod et al., 1996).

expressed in the anterior PSM and its expression is regulated by Hes7, but not by Mesp2 (data not shown). There may be different gene regulatory mechanisms along the AP axis in the PSM, where the cyclic expression in the posterior PSM must be regulated by a clock mechanism, but expression in the anterior PSM may also be regulated by a different mechanism under the control of Mesp2. The link between these two mechanisms is currently unknown but the clock mechanism which is operated by both Wnt and Notch signaling may affect Mesp2 regulation.

nkd is a *Drosophila* segment polarity gene that encodes an antagonist of the Wnt signal, Wingless. It is known that mouse Nkd1 directly binds to Dishevelled (Dvl) (Wharton et al., 2001) and that Nkd1 antagonizes Wnt signaling in both *Xenopus* (Yan et al., 2001a) and *Drosophila* (Wharton et al., 2001) embryos. Thus, it is possible that the Nkd1 protein also functions as an antagonist of Wnt signaling in the PSM. Of great interest, therefore, is the exact function of Nkd1 during somitogenesis. Using luciferase reporter assays, it has previously been demonstrated that *Axin2* is directly upregulated by Wnt/β-catenin signaling (Aulehla et al., 2003). One possibility, therefore, for the function of Nkd1 is that, in mouse, it suppresses the transcription of *Axin2* by antagonizing the Wnt signals, and hence, Nkd1 oscillations may contribute to the oscillatory expression of *Axin2*. To further address this question, it will be necessary to analyze the distribution of the Nkd1 protein and examine whether it has a sufficiently high turnover to generate such oscillatory expression patterns. However, both Axin and Nkd1 have been demonstrated to bind Dvl via its PDZ domain (Li et al., 1999; Seidensticker and Behrens, 2000; Yan et al., 2001a; Wharton et al., 2001). Hence, if Axin2 can bind Dvl with a similar affinity as Axin, it may compete with Nkd1 for Dvl binding to initiate distinct functions. Alternatively, they may work co-operatively to suppress Dvl. The binding to Dvl not only affects Wnt signaling but also may affect Notch signaling since it has already been shown to bind the processed form of Notch receptor (NICD) (Axelrod et al., 1996). It has also been reported that Dvl1/Dvl2 double knockout embryos exhibit defects in somite segmentation (Hamblet et al., 2002), which may suggest that regulation of Wnt signaling via Axin2 and /or Nkd1 is involved in segmental patterning. The link between Wnt and Notch signaling remains one of the central unsolved mechanisms underlying both the generation and regulation of the segmentation clock. Since *nkd1* oscillation is

disrupted in Hes7-null mutants, there may be a negative feed-back loop between the Notch and Wnt signaling pathways and it is likely that their interaction is essential for the establishment of the molecular clock required for accurate somitogenesis (Fig. 6B).

4. Experimental procedures

4.1. Experimental animals

Heterozygous Mesp2-mutant (*Mesp2*$^{+/-}$) mice were intercrossed to obtain either homozygous, heterozygous or wild-type embryos in the same litter. The appearance of vaginal plugs was designated as embryonic day 0.5 (dpc). Homozygous Mesp2-null embryos were identified by a no segmentation phenotype, as described previously (Saga et al., 1997). Hes7-knockout mice and Dll1-knockout mice were kindly provided by Yasumasa Bessho (Kyoto Univ. Japan) and Achime Gosller (Hanover Univ. Germany), respectively. Samples of *vt/vt* embryos were provided by Shinji Takada (National Institute for Basic Biology, Japan).

4.2. Preparation of subtractive cDNA libraries

As shown in Fig. 1, total RNA isolates were prepared from two pieces of the dissected portions of either wild-type (WP: PSM of wild-type and WS: somite region of wild-type) or homozygous (HPS: PSM of Mesp2-null) 11.5 dpc embryos using Isogen (Nippon Gene, Japan). We utilized the subtractive hybridization method of Kaneko-Ishino et al. (1995) with slight modifications. Briefly, to generate tester cDNAs for subtraction, cDNAs were synthesized and amplified with the Smart cDNA Amplification Kit (Clontech, USA), whereas the driver cDNAs were generated using a cDNA Synthesis Kit (TaKaRa, Japan). Amplification of driver cDNAs was performed using a ligated linker (P-linker). A biotinylated P-primer, complementary to the P-linker, was used for the subsequent PCR (Kaneko-Ishino et al., 1995).

PCR primer 5′-AAGCAGTGGTAACAACGCAGAGT-3′.
P-linker 5′-GATTACTCGAGACTAATATC-3′;
5′-pGATATTAGTCTCGAGTA-3′.

We constructed two libraries; one generated by subtraction between [WP] (tester) and [WS] (driver) to enrich for PSM genes, and a second synthesized by subtraction between [WP] (tester) and [HPS] (driver) to enrich for possible Mesp2 downstream genes. Tester cDNA samples (60 ng) were hybridized to biotinylated driver cDNAs (1.2 μg) (the ratio was increased to 1:100 in the case of the second and third subtractions). After ethanol precipitation, total cDNAs were subjected to absorption by 4 mg of Dynabeads M-280 conjugated to streptavidin (Dynal). The remaining cDNAs were amplified by PCR. One half of each sample was used for the subsequent subtraction steps, and the cDNA isolates that were obtained after three consecutive subtractions and PCR amplifications were cloned into the pCRII vector using a TA Cloning Kit (Invitrogen). We designated these subtractive libraries as the PTS library for [WP] and [WS], and as the ST library for [WP] and [HPS].

4.3. Sequencing and computer analysis

cDNA clones that were randomly selected from both of the subtractive libraries that we generated, 406 and 636 clones from PTS and ST, respectively, were partially sequenced and analyzed using the BLAST program through the National Library of Medicine and Celera databases. Only novel clones were further screened by ISH. The numbers of selected clones were 251 and 280, for PTS and ST, respectively.

4.4. Screening by in situ hybridization

The basic method used for whole mount ISH has been described previously by our laboratory (Saga et al., 1996), although for this study automated ISH was employed (InsituPro, M&S Instruments Trading Inc. Japan). The tail regions, containing both somite and PSM of 11.5 dpc embryos, were used in these analyses. RNA probes were generated by transcription, with either T7 RNA polymerase or SP6 RNA polymerase, of template DNAs prepared by PCR using M13 specific sites within the vector. M13 reverse primer: 5′-CAGGAAACAGCTATGAC-3′; M13 forward primer: 5′-GTAAAACGACGGCCAG-3′.

4.5. Whole-mount in situ hybridization probes

A BamHI-PstI 429 bp fragment of *nkd1* cDNA was used as an *nkd1* exon probe. As *nkd1* intron probes, we utilized a mixture of two genomic fragments; the XbaI 1.3 kb fragment in intron 4 and the NcoI 1.4 kb fragment in intron 6. For *Axin2* hybridization, we amplified a 1360 bp PCR fragment from the coding region of this gene from a PSM cDNA library (Ohara et al., 2002) using the forward primer 5′-GTCTGGAGGAGCGGCTGCAGCAGATCCG-3′ and reverse primer 5′-CAAGGCTCAGTCGATCCTCTC-CACTTTGC-3′.

4.6. In vitro explant cultures

In vitro explant culture was performed as previously described (Takahashi et al., 2000). The tail regions of ICR embryos (11.5 dpc) were bisected at the midline with either a tungsten or ground sewing needle. One half was fixed immediately and the other was cultured for either 60 or 120 min. After culturing, specimens were fixed and used for subsequent ISH analysis.

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References

Aulehla, A., Wehrle, C., Brand-Saberi, B., Kemler, R., Gossler, A., Kanzler, B., Herrmann, B.G., 2003. Wnt3a plays a major role in the segmentation clock controlling somitogenesis. Dev. Cell 4, 395–406.

Axelrod, J.D., Matsuno, K., Artavanis-Tsakonas, S., Perrimon, N., 1996. Interaction between Wingless and Notch signaling pathways mediated by dishevelled. Science 271, 1826–1832.

Behrens, J., Jerchow, B., Würtele, M., Grimm, J., Asbrand, C., Wirtz, R., et al., 1998. Functional interaction of an axin homolog, conductin, with β-catenin, APC, and GSK3β. Science 280, 596–599.

Bessho, Y., Sakata, R., Komatsu, S., Shiota, K., Yamada, S., Kageyama, R., 2001. Dynamic expression and essential functions of Hes7 in somite segmentation. Genes Dev. 15, 2642–2647.

Bessho, Y., Hirata, H., Masamizu, Y., Kageyama, R., 2003. Periodic repression by the bHLH factor Hes7 is an essential mechanism for the somite segmentation clock. Genes Dev. 17, 1451–1456.

Dubrulle, J., McGrew, M.J., Pourquie, O., 2001. FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal Hox gene activation. Cell 106, 219–232.

Greco, T.L., Takada, S., Newhouse, M.M., McMahon, A.P., Camper, S.A., 1996. Analysis of the vestigial tail mutation demonstrates that Wnt-3a gene dosage regulates mouse axial development. Genes Dev. 10, 313–324.

Hamblet, N.S., Lijam, N., Ruiz-Lozano, P., Wang, J., Yang, Y., Luo, Z., et al., 2002. Dishevelled 2 is essential for cardiac outflow tract development, somite segmentation and neural tube closure. Development 129, 5827–5838.

Hsu, S., Galceran, J., Grosschedl, R., 1998. Modulation of transcriptional regulation by LEF-1 in response to Wnt-1 signaling and association with β-catenin. Mol. Cell Biol. 18, 4807–4818.

Kaneko-Ishino, T., Kuroiwa, Y., Miyoshi, N., Kohda, T., Suzuki, R., Yokoyama, M., et al., 1995. Peg1/Mest imprinted gene on chromosome 6 identified by cDNA subtraction hybridization. Nature Genet. 11, 52–60.

Li, L., Yuan, H., Weaver, C.D., Mao, J., Farr III., G.H., Sussman, D.J., et al., 1999. Axin and Frat1 interact with dvl and GSK, bridging Dvl to GSK in Wnt-mediated regulation of LEF-1. Eur. Mol. Biol. Org. J. 18, 4233–4240.

Liu, Y., Jiang, H., Crawford, H.C., Hogan, B.L., 2003. Role for ETS domain transcription factors Pea3/Erm in mouse lung development. Dev. Biol. 261, 10–24.

Nomura-Kitabayashi, A., Takahashi, Y., Kitajima, S., Inoue, T., Takeda, H., Saga, Y., 2002. Hypomorphic Mesp allele distinguishes establishment of rostrocaudal polarity and segment border formation in somitogenesis. Development 129, 2473–2481.

Ohara, O., Nagase, T., Mitsui, G., Kohga, H., Kikuno, R., Hiraoka, S., et al., 2002. Characterization of size-fractionated cDNA libraries generated by the in vitro recombination-assisted method. DNA Res. 9, 47–57.

Palmeirim, I., Henrique, D., Ish-Horowicz, D., Pourquié, O., 1997. Avian hairy gene expression identifies a molecular clock linked to vertebrate segmentation and somitogenesis. Cell 91, 639–648.

Porfiri, E., Rubinfeld, B., Albert, I., Hovanes, K., Waterman, M., Polakis, P., 1997. Induction of a β-catenin-LEF-1 complex by wnt-1 and transforming mutants of β-catenin. Oncogene 15, 2833–2839.

Pourquié, O., 2001. Vertebrate somitogenesis. Annu. Rev. Cell Dev. Biol. 17, 311–350.

Rasmussen, J.T., Deardorff, M.A., Tan, C., Rao, M.S., Vetter, M.L., 2001. Regulation of eye development by frizzled signaling in Xenopus. Proc. Natl Acad. Sci. USA 98, 3861–3866.

Ristevski, S., Tam, P.P., Hertzog, P.J., Kola, I., 2002. Ets2 is expressed during morphogenesis of the somite and limb in the mouse embryo. Mech Dev. 116, 165–168.

Saga, Y., Hata, N., Kobayashi, S., Magnuson, T., Seldin, M., Taketo, M.M., 1996. MesP1: a novel basic helix-loop-helix protein expressed in the nascent mesodermal cells during mouse gastrulation. Development 122, 2769–2778.

Saga, Y., Hata, N., Koseki, H., Taketo, M.M., 1997. Mesp2: a novel mouse gene expressed in the presegmented mesoderm and essential for segmentation initiation. Genes Dev. 11, 1827–1839.

Sawada, A., Shinya, M., Jiang, Y.J., Kawakami, A., Kuroiwa, A., Takeda, H., 2001. Fgf/MAPK signalling is a crucial positional cue in somite boundary formation. Development 128, 4873–4880.

Schmidt, C., Christ, B., Maden, M., Brand-Saberi, B., Patel, K., 2001. Regulation of Epha4 expression in paraxial and lateral plate mesoderm by ectoderm-derived signals. Dev. Dyn. 220, 377–386.

Seidensticker, M.J., Behrens, J., 2000. Biochemical interactions in the wnt pathway. Biochem. Biophys. Acta 1495, 168–182.

Takada, S., Stark, K.L., Shea, M.J., Vassileva, G., McMahon, J.A., McMahon, A.P., 1994. Wnt-3a regulates somite and tailbud formation in the mouse embryo. Genes Dev. 8, 174–189.

Takahashi, Y., Koizumi, K., Takagi, A., Kitajima, S., Inoue, T., Koseki, H., Saga, Y., 2000. Mesp2 initiates somite segmentation through the Notch signalling pathway. Nature Genet. 25, 390–396.

Takahashi, Y., Inoue, T., Gossler, A., Saga, Y., 2003. Feedback loops comprising Dll1, Dll3 and Mesp2, and differential involvement of Psen1 are essential for rostrocaudal patterning of somites. Development 18, 4259–4268.

Tan, C., Deardorff, M.A., Saint-Jeannet, J.P., Yang, J., Arzoumanian, A., Klein, P.S., 2001. Kermit, a frizzeled interacting protein, regulates frizzeled 3 signaling in neural crest development. Development 128, 3665–3674.

Wharton Jr., K.A., Zimmermann, G., Rousset, R., Scott, M.P., 2001. Vertebrate proteins related to Drosophila Naked Cuticle bind Dishevelled and antagonize Wnt signaling. Dev. Biol. 234, 93–106.

Yamamoto, H., Flannery, M.L., Kupriyanov, S., Pearce, J., McKercher, S.R., Henkel, G.W., et al., 1998. Defective trophoblast function in mice with a targeted mutation of Ets2. Genes Dev. 12, 1315–1326.

Yan, D., Wallingford, J.B., Sun, T.Q., Nelson, A.M., Sakanaka, C., Reinhard, C., et al., 2001a. Cell autonomous regulation of multiple Dishevelled-dependent pathways by mammalian Nkd. Proc. Natl Acad. Sci. USA 98, 3802–3807.

Yan, D., Wiesmann, M., Rohan, M., Chan, V., Jefferson, A.B., Guo, L., et al., 2001b. Elevated expression of axin2 and hnkd mRNA provides evidence that Wnt/β-catenin signaling is activated in human colon tumors. Proc. Natl Acad. Sci. USA 98, 14973–14978.

Zeng, W., Wharton Jr., K.A., Mack, J.A., Wang, K., Gadbaw, M., Suyama, K., et al., 2000. Naked cuticle encodes an inducible antagonist of Wnt signalling. Nature 403, 789–795.

"POINTS TO CONSIDER" REGARDING SAFETY ASSESSMENT OF BIOTECHNOLOGY-DERIVED PHARMACEUTICALS IN NON-CLINICAL STUDIES (ENGLISH TRANSLATION)

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ABSTRACT — Regulatory and industrial scientists collaborated to publish a "points to consider" document regarding the safety assessment of biotechnology-derived pharmaceuticals in non-clinical studies in 2002 (Pharmaceutical Non-clinical Investigation Group, 2002). The collaboration team intended to clarify the interpretation of ICH-S6 guideline and furthermore share recent Japanese practices on this matter. However, the document was written in Japanese. Thus, we share here an English translation of the document so that non-native Japanese correctly understand the contents.

KEY WORDS: English translation, Points to consider, Non-clinical, Biotechnology-derived pharmaceuticals, ICH-S6 guideline

INTRODUCTION

Scientists from National Institute of Health Sciences, Pharmaceuticals and Medical Devices Evaluation Center (currently, Pharmaceuticals and Medical Devices Agency) and Japan Pharmaceutical Manufacturers Association collaborated to publish a "points to consider" document regarding the safety assessment of biotechnology-derived pharmaceuticals, or biopharmaceuticals, in non-clinical studies in 2002 (Pharmaceutical Non-clinical Investigation Group, 2002). The collaboration team intended to clarify the interpretation of ICH-S6 guideline and furthermore share recent Japanese practices on this matter. However, it was written in Japanese. Thus, the follow-up team (the authors of this review and almost the same as the initial collaboration team members) made an English translation of the document and collected comments on it from experts in the US and EU. The experts were generally very supportive of the ideas shown in the "points to consider" document. They also suggested more clarification on some other ideas. Considering those comments, the follow-up team has revised the Japanese "points to consider" document to be published in the near future and shares here the English translation of the revised document so that non-native Japanese correctly understand the contents. The following sections 1 to 15 are the English translation.

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Background

Biotechnology-derived pharmaceuticals (biopharmaceuticals) were initially developed in the 1980s. In recent years, various kinds of new biopharmaceuticals suitable for clinical trials and marketing are on the increase.

A primary objective of the development of biopharmaceuticals derived from animal or plant tissue is to have proteins consisting of primary amino acid sequence which are identical to endogenous human proteins via DNA recombinant technology. Human proteins are expected to have low or no antigenicity and the potential for long-term use in humans. On the other hand, it is recognized that human protein biopharmaceuticals may sometimes be immunogenic in animals due to differences in the primary amino acid sequence of the protein between humans and animals; however, this is not always the case. There are many examples of human proteins produced by recombinant DNA technology that are not immunogenic in animals. In cases where a human protein is highly immunogenic in animals, the safety evaluation of human proteins in animals sometimes has technical limitations.

In addition, differences in approaches for safety evaluation of biopharmaceuticals among the European Union, the United States and Japan have been identified, pointing out the need for harmonization between the three regions. In 1997 the three regions reached an agreement concerning "Preclinical safety evaluation of biotechnology-derived pharmaceuticals" ("ICH S6 guideline"). Based on this ICH-S6 guideline, the Notification No. 326 was issued, as ICH Step 5, by the Ministry of Health and Welfare (MHW) in 2000. The interpretation described here is related to this notification, and was developed in conformity with the ICH guideline, serving as a reference for the data gathered after issuance of the notification.

The guideline employs the principle that preclinical safety evaluation of biopharmaceuticals should be addressed on a case-by-case basis. The term "case-by-case" refers to the understanding that (1) as biopharmaceuticals intended as human-specific products are developed, safety evaluation in animals has limitations and cannot be addressed by routine nonclinical safety study methods adapted to chemically synthesized non-protein/peptide compounds (CSC), and (2) not only human-type protein and peptides but also various modified proteins and monoclonal antibody biopharmaceuticals are being developed and therefore study design optimized to each type of biopharmaceutical is needed.

Difficulty in conducting safety evaluation of biopharmaceuticals by routine studies

A major consideration for safety testing of biopharmaceuticals is that they can possess highly selective pharmacological actions and/or species selectivity as opposed to CSC, and therefore safety evaluation of biopharmaceuticals in animals is difficult. Some biopharmaceuticals are active only in humans. Therefore, testing these molecules in irrelevant animals is of no predictive value with respect to human safety. If the proteins are highly immunogenic and if the immune response is "neutralizing", then the conduct of repeat-dose studies beyond 2-3 weeks duration is problematic, especially if dose schedule is episodic compared to the daily dosing regimen.

Type of biologics and scope of the guideline

1. Biopharmaceuticals: protein/peptide based biologics

Biopharmaceuticals covered by the guideline include protein/peptide products consisting of amino acids. The upper column of Table 1 shows the subcategories of biopharmaceuticals. Antibodies are originally classified by protein subcategory, but a separate category was provided for antibodies because the biological action differs from general protein products. In recent years, the development of human protein analogs has sporadically been observed with improvement in efficacy expected, and therefore approaches to these analogs are also described here. The guideline should cover safety evaluation of biopharmaceuticals by taking into account the type and clinical application of individual biopharmaceuticals. The considerations for each type of biopharmaceutical are shown below. The safety of impurities and degradation products in biopharmaceuticals need to be comprehensively assessed, according to their quality and bioactivity.

1) Proteins

When a human-type protein is used at a blood concentration exceeding the physiological level, studies for safety evaluation should be designed with reference to many of the considerations mentioned in the guideline. Moreover, consideration should be given to entirely different physiological secretion patterns in humans. Changes in blood concentration are known to be more significant than the concentration level itself for some classes of proteins. Biopharmaceuticals intended for sustained-release profile show changes in blood concentration level further diverging from the physiological secretion pattern. Therefore, when the changes in blood concentration of exogenous human

protein differ from the physiological secretion patterns of endogenous proteins, attention should be paid to the potential changes in physiological action.

For animal proteins or human-type protein analogs consisting of natural amino acids (*i.e.* human-type protein analogs with original human protein amino acids being substituted with other natural amino acids, added natural amino acids to or deleted amino acids), consideration should be given to the potential difference in potency and quality of biological activity from those of original human protein. For example, in a human-type protein analog where the substituted site is involved in receptor recognition sites, its biological activity would be enhanced/diminished or new biological activity may occur. Moreover, depending on the type and site of amino acid replaced, a new antigen determinant (epitope) may express and result in changes of antigenicity.

For human-type protein analogs with non-natural amino acids, in addition to the above considerations, attention should be paid to the potential biological activity and its pharmacokinetic behavior in the fragment containing the site where this protein has been metabolized. For example, no genotoxicity studies are needed for proteins not passing through cell membranes, while applicability of this theory should be discussed on a case-by-case basis for fragments containing non-natural amino acids. No metabolism studies are needed for proteins just degraded into amino acids, while metabolism studies may provide useful information for proteins containing non-natural amino acids.

Two types of bioconjugates may exist. Bioconjugates of human-type protein with other protein may have the combined biological activity of both proteins and their effects on the body may be altered due to the interaction between these proteins. Therefore, consideration should be given to the conducting of safety evaluation in pharmacological studies. On the other hand, bioconjugates of human-type protein with

Table 1. Type of biopharmaceuticals and scope of the guideline.

Biopharmaceuticals: protein/peptide based biologics (covered by the guideline)		
	Proteins	
		Human-type protein
		Non-human protein
		Human-type protein analog consisting of natural amino acid
		Human-type protein analog containing non-natural amino acid
		Bioconjugate of human-type protein and other protein
		Bioconjugate of human-type protein and organic linker
	Peptides	
		Human-type peptide
		Non-human-type peptide
		Human-type peptide analog consisting of natural amino acid
		Human-type peptide analog containing non-natural amino acid
	Antibodies	
		Monoclonal antibodies/chimera antibodies
		Immunoconjugates
Related biopharmaceutical: non-protein/peptide based biopharmaceuticals (not covered by the guideline but its basic principles can be used as reference)		
	Peptide mimic (CSC having a selective affinity to human peptide receptors)	
	Gene therapy	
		Anti-sense compounds
		Ribozyme
Other biopharmaceuticals (not covered by the guideline and safety evaluation conforms to other standards)		
	Antibiotics	
	Allergen extracts	
	Vitamins	
	Viral vaccines, etc.	

organic linker can be handled in a manner similar to human-type protein analogs containing non-natural amino acid.

2) Peptides

Peptides, like proteins, consist of amino acids, although their molecular weights are smaller than those of proteins. Therefore, the above considerations for proteins are also applicable to peptides. Antibody formation, which is a key issue in animal experiments for human-type proteins, is generally dependent on molecular weight (*i.e.* probability of antibody formation is lower if molecular weight is lower). The guideline covers not only biotechnologically produced peptides but also chemically synthesized peptides.

3) Antibodies

Antibodies are usually targeted to specific receptors, especially in the case of monoclonal antibodies. Many of these antibodies are inherently species-specific. It is important for the sponsor to verify species specificity in order to justify the use (or non-use) of a particular animal species, for safety studies. In the cases where an appropriate animal model is not available, then the use of homologous antibodies for animals or the use of relevant transgenic animals expressing the human antigen should be considered. In addition, when an IgG antibody is used in possibly pregnant or lactating women and based upon the intended indication, reproductive toxicity needs to be investigated because of its potential for antibody being transferred to placenta or the milk. Immunoconjugates of antibodies, conjugated either with other proteins or with organic linker, should be handled the same way as bioconjugates as described above.

2. Related biopharmaceuticals: non-protein/peptide based biologics

The middle section of Table 1 shows the pharmaceuticals not classified as biopharmaceuticals, although having selective pharmacological action similar to biopharmaceuticals. Their safety evaluation in animals is sometimes difficult. The guideline does not cover these pharmaceuticals but its basic principles can be used as reference.

1) Peptide mimics

Peptide mimics are CSC with a selective affinity to peptide receptors. When a peptide mimic to be tested has an action specific to human peptide receptors, sufficient response may not be obtained in those animal species that have been commonly used in toxicity studies. In such cases, appropriate safety evaluation may be feasible by designing the study, using the basic principles of the guideline for biopharmaceuticals as a reference.

2) Antisense compounds and ribozymes

Antisense compounds and ribozymes used for gene therapy need to be examined separately from biopharmaceuticals. They selectively modify (generally suppress) expression of a certain kind of gene, for example suppressed production of endogenous molecules by a certain kind of gene, thereby exerting their efficacy. Therefore, as in the case of antibodies, if the antisense compound or ribozyme does not recognize or interact with a gene in the test animal, then such studies would not predict human safety concerns. In such cases, the safety evaluation would be incorporated into primary pharmacodynamic studies in relevant animal models. One should know the limitation of nonclinical evaluation for antisense compounds and ribozymes.

3. Other biologics

The conventional biologics shown in the bottom section of Table 1 are not covered by this guideline. Non-clinical safety evaluation based on relevant standards is necessary for them.

Selection of animal species

Studies conducted with animals that do not respond pharmacologically to a test biopharmaceutical do not provide useful safety information. Similarly, in the case that a biopharmaceutical produces significant neutralizing antibodies in an animal, it may be difficult to evaluate the safety of the compound in the animal study. If no relevant animal species are available, animal models mimicking the human disease, transgenic animals expressing human proteins (*e.g.* receptors) or homologous proteins (animal) may be useful. If neither of them is available, the necessity of toxicity studies including cardiovascular and respiratory function tests in a single animal species (*e.g.* 14-day repeated-dose toxicity study) should be justified on a case-by-case basis.

Setting of the maximum dose

In many cases, no toxicity is observed in safety studies with biopharmaceuticals, which have aroused much discussion concerning a maximum dose for toxicology studies. ICH-S6 Expert Working Group suggested that the maximum dose of a biopharmaceutical could be about 5 times higher than the intended maxi-

mum clinical dose of the biopharmaceutical based on its AUC. Another thought is that the maximum dose may be taken as the dose in which the pharmacodynamic response has reached the plateau (pharmacodynamic maximum dose). There is generally no need to investigate biopharmaceuticals at doses much higher than the intended clinical dose, unlike CSC.

In cases of effects at higher doses of biopharmaceuticals, one must note the potential association of protein molecules as a result of the high protein concentration. The difference of the dosing formulation between associated molecules and monomers in biopharmaceuticals might affect the pharmacokinetics and pharmacodynamics. Some biopharmaceuticals are not absorbed from subcutaneous regions to the blood in associated form and can be absorbed only after being dissociated to a monomer or dimer.

Toxicological effects and pharmacological action

In many cases, only exaggerated pharmacological effects are observed in toxicology studies with biopharmaceuticals. Sometimes these effects are difficult to distinguish from compound-related toxicity. However, if the effect is related to mechanism (predictable) and reversible, it should not be considered as an adverse effect.

In the case that lethality is observed, it should still be prudent to assess whether the death is due to toxicity or an exaggerated pharmacological effect, considering the clinical application. For example, death due to hemorrhage is sometimes observed in healthy animals after administration of biopharmaceuticals with an anti-coagulative activity. In addition, death due to hypoglycemia can occur in healthy animals after administration of insulins. To attribute these death cases to toxicity has little value for determining human safety in clinical practice. Because these changes are observed only in healthy animals, and because biopharmaceuticals are prescribed for the normalization of abnormal functions in patients (*e.g.* hypercoagulability or hyperglycemia) through their pharmacological actions, one can easily assume that hemorrhage or hypoglycemia due to excessive expression of the pharmacological actions may occur.

Safety pharmacological studies

Safety pharmacological studies of biopharmaceuticals are performed as a separate study or incorporated in the design of toxicity studies in order to examine the effects on vital functions. Normally, follow-up or supplementary studies are performed to provide a greater depth of understanding than that provided by the "core battery" (central nervous system, circulatory system and respiratory system) and renal system. *In vitro* electrophysiological studies are not applicable for biopharmaceuticals. This is because CSC acts on each cellular channel after passing through the cell membranes, while biopharmaceuticals are not expected to act in the same manner because they cannot pass through cell membranes. A part of or all of the safety pharmacological studies can be obviated in the case of biopharmaceuticals whose mechanism of actions is highly selective.

The safety pharmacological studies should be designed under the same considerations as the toxicity studies as regards selection of animal species, dose and species specificity.

ADME studies (absorption, distribution, metabolism and excretion)

As stated in the guideline, it is difficult to establish uniform guidelines for ADME studies for the various types of biopharmaceuticals. ADME study should be designed on a case-by-case basis, with the following considerations.

The patterns of drug absorption may be influenced by formulation, concentration, administration site, and/or volume. ADME studies should, whenever possible, utilize preparations that are representative of those actually intended for toxicological studies and clinical use and employ a route of administration that is relevant to the anticipated clinical trials. In addition, an assessment of systemic exposure should be performed together with toxicity studies.

When using radiolabeled proteins, it is important to show that the radiolabeled test material maintains activity and biological properties equivalent to that of the unlabeled material, and to consider the stoichiometric radiolabeling, the loss of radiolabel, recycling of radiolabeled amino acid into non-drug related protein and aggravation of stability. However, biopharmaceuticals not meeting the satisfactory radiolabeling should inevitably be evaluated using unlabeled proteins in many cases. Degradation of protein to peptides and amino acid moiety is commonly expected as a representative metabolic pattern. Therefore, conventional biotransformation studies are not needed for biopharmaceuticals consisting of natural amino acid. However, metabolism studies of biopharmaceuticals containing non-natural amino acid may provide useful information. In such cases, radiolabeled proteins should be prepared in order to trace the pharmacokinetic behav-

ior of the non-natural amino acid fragments. When using ^{125}I radiolabeled proteins in distribution studies, consideration should be given to the formation of inorganic iodine by deiodinization *in vivo*. For example, free ^{125}I thus produced is accumulated in the thyroid gland, and thereby the biopharmaceutical seems to be distributed mainly in the thyroid gland.

For biopharmaceuticals, particular attention should be paid to expression of neutralizing antibodies. However, neutralizing antibodies may appear as a result of not only repeated administration but also by single administration depending on the pharmacokinetic properties of the biopharmaceuticals, and therefore particular attention should be paid to the antibodies or to the PK.

Single-dose toxicity studies

An objective of single-dose studies is to define the relationship of dose to systemic and/or local toxicity. For biopharmaceuticals repeatedly administered clinically, the data from single-dose toxicity studies can be used to select doses for repeated-dose toxicity studies. For biopharmaceuticals with a weak toxicity, repeated-dose toxicity studies can be performed without conducting single-dose toxicity studies under the GLP condition, and therefore single-dose toxicity studies in two animal species is not considered to be as necessary for these biopharmaceuticals than it is for CSC.

When necessary, single-dose toxicity can be evaluated as a component of safety pharmacology or primary pharmacodynamic studies using animal models. When provided doses set for the repeated-dose toxicity study are reasonable, the initial administration data in the repeated-dose toxicity study can be used as data for single-dose toxicity.

Since biopharmaceuticals need not be examined at high doses such as the approximate lethal dose, conducting single-dose toxicity studies merely to obtain information on the potential of toxic substances, etc. would be meaningless. Single-dose toxicity studies in non-rodents should in general only be considered in cases where rodents are not considered a relevant species.

Repeated-dose toxicity study

The duration of repeated-dose toxicity studies should be based on the intended duration of clinical exposure and disease indication as follows.

1) In cases of biopharmaceuticals intended for short-term use (*e.g.* $\leq$ 7 days) and/or acutely life-threatening diseases, repeated-dose toxicity studies up to two weeks have been considered adequate to support clinical studies as well as obtaining marketing authorization.
2) In the case those biopharmaceuticals are intended for chronic indications, studies of 6 months have generally been accepted as regulatory agreement, although in some cases shorter or longer durations have been adequate to support marketing authorizations. The duration of long-term toxicity studies should be scientifically justified.
3) In cases of biopharmaceuticals not covered by 1) or 2), the duration for animal dosing should be determined based on the intended clinical duration, *i.e.* generally 1-3 months.

Typically, toxicity studies are performed in two animal species. However, for toxicity studies where there is only one relevant animal species, the studies may be performed in a single animal species. When two animal species show the same toxicity profile in short-term studies, long-term studies may employ one animal species. Comparison of toxicity profiles means comparing the type and severity of any toxicity observed. However, biopharmaceuticals with a weak toxicity may display no toxicity at high doses in some cases. Therefore, even if no toxicity is observed in any animal species, the same toxicity profile can be determined based on the justified rationale for determining the maximum dose.

The basic concepts of inclusion of toxicokinetics and setting of recovery period are identical to the concepts for designing usual repeated-dose toxicity studies. For biopharmaceuticals that induce prolonged pharmacological/toxicological effects, recovery group animals should be monitored until reversibility is demonstrated. However, for mechanisms of change whose toxic action is clear and whose reversibility is predictable, recovery studies are not needed. Specifically for biopharmaceuticals; alteration may be observed due to the excessive expression of pharmacological actions; therefore, the pharmacological assessment should be made for potential reversibility of the alteration observed. In addition, whether the alteration accompanies organic changes or not may be useful for assessing potential reversibility. When the mechanism of the toxic effect is unknown or a unique mechanism obviously different from the pharmacological actions is involved, recovery studies should be performed.

Particular attention should be paid to appearance of neutralizing antibodies, but the detection of neutralizing antibodies should not be the sole criterion for early termination, or not conducting studies. In some

cases, useful studies can be achieved by prudently performing them. The early termination of studies should be considered when pharmacological actions are masked and no biological response is observed due to the occurrence of unexpected adverse toxicity attributable to antibody formation or marked decrease in plasma concentrations of test materials during the study period. When the results from short-term studies or preliminary studies suggest the potential of any such situation in longer-term repeated dose toxicity studies, to conduct longer-term studies should prudently be considered for assuring the significance of safety evaluation.

Antigenicity and immunotoxicity studies

These are important assessment because the objective of antigenicity studies is the prediction of anaphylactic shock. For biopharmaceuticals, however, there are no appropriate animal models that are considered to be predictive of human allergic responses. Although technical progress would be achieved in the future, antigenicity should be evaluated carefully in clinical practice at present.

Many biopharmaceuticals are intended to either stimulate or suppress the immune system of the host. When biopharmaceuticals with no expected immunopharmacological action affect the immune system, immunotoxicity studies should be performed. However, standard immunotoxicity assessments as currently being discussed for CSCs are not appropriate. In such cases, repeated-dose toxicity studies in relevant animal species may clarify the effects on the immune system.

Reproductive and developmental toxicity studies

Reproductive and developmental toxicity studies are needed when 1) relevant animal species exist, 2) application for pregnant or women of childbearing potential (WOCBP) is intended, and 3) natural biopharmaceuticals do not exist or structurally differ from the biopharmaceuticals.

When standard reproductive and developmental toxicity studies are unfeasible due to neutralizing antibody formation, etc., although it is deemed necessary, the study design and dosing schedule may be modified based on issues related to species specificity, antigenicity, biological activity and/or a long elimination half-life. For example, a reproductive and developmental toxicity study with shorter periodic dosing than the whole period dosing shown in the toxicology guideline for CSC can be meaningful. In addition, alternative studies using relevant transgenic animals, or homologous proteins, should be considered. However, as reproductive performance in transgenic animals has not yet been clarified, careful selection is therefore needed for the study system.

The need for reproductive and developmental toxicity studies is dependent upon the clinical indication and intended patient population. For example, when 1) no relevant animal species exist and the biopharmaceutical is not indicated for pregnant or WOCBP, 2) structurally comparable to a natural biopharmaceutical for which there is wide experience in clinical practice and 3) the biopharmaceutical is indicated for patients without childbearing potential and indicated for those with serious diseases, reproductive and developmental toxicity studies can be obviated.

Points to consider on the need for assessment of reproduction toxicity of human insulin analogues have been published (CPMP, 2002).

Genotoxicity studies

This is generally not applicable for biopharmaceuticals to routinely implement the genotoxicity studies required for CSC. Proteins and peptides are not expected to interact directly with DNA or other chromosomal material by passing through the cell membrane. On the other hand, as the guideline describes "With some biopharmaceuticals there is a potential concern about accumulation of spontaneously mutated cells (*e.g.*, via selectively facilitating a predominating factor of proliferation) leading to carcinogenicity, alternative *in vivo* or *in vitro* models to address such concerns may have to be developed and evaluated." When *in vitro* or *in vivo* data suggest the potent biopharmaceuticals' ability to strongly stimulate cell proliferation, conducting carcinogenicity studies should be considered. In the case of human-type proteins or peptides, it would be helpful for assessing the necessity of further studies to compare the physiological concentration of the biopharmaceutical in blood or tissue with the concentration at which the enhancement activity of the biopharmaceutical on cell proliferation is observed. Human protein analogs should be evaluated for potential difference in activity from human protein.

Genotoxicity studies should be performed for biopharmaceuticals with bioconjugates having an organic linker molecule or biopharmaceuticals with non-natural amino acid. The need for genotoxicity studies depends on whether a biopharmaceutical is a natural human protein or an analog such as a bioconjugate. Genotoxicity studies are not needed for natural

proteins, because it is not expected that they would interact directly with DNA or other chromosomal material after passing through the membrane and then just degraded into natural amino acid. In the case of analogs, it may be necessary to assess their genotoxicity under metabolic activation conditions, since there might be a possibility that unknown compounds can potentially be formed. However, genotoxicity studies can be obviated even for analogs by demonstrating no penetration into cells using radiolabeled materials.

Carcinogenicity studies

The guideline requires flexible approaches to evaluate the carcinogenic risk of biopharmaceuticals under the description that "Standard carcinogenicity studies are generally inappropriate for biopharmaceuticals. However, product-specific assessment of carcinogenic potential may still be needed depending upon duration of clinical dosing, patient population and/or biological activity of the product (*e.g.*, growth factors, immunosuppressive agents, etc.) When there is a concern about carcinogenic potential, a variety of approaches should be considered to evaluate the risk."

If the following are confirmed, generally, carcinogenicity studies are not necessary even when a biopharmaceutical is used for a long period of time.

1) It is used for substitution therapies at the physiological level.
2) It has no physiological activity differing from that of endogenous substances.
3) Its biological action is not significantly stronger than that of endogenous substances.
4) It has no potential to induce tumor cell division (in case of growth promoter).
5) It neither locally retains nor accumulates at a high concentration for a long period of time.
6) It does not have sustained pharmacological action.
7) In repeated-dose toxicity studies when a dosing duration adequate for evaluation is attained, no preneoplastic lesions are observed.
8) The results of genotoxicity studies were negative in the case that the studies had been conducted (*e.g.*, bioconjugate with organ linker).

Conducting carcinogenicity studies should be considered in some cases due to the dosing duration, relationship of target diseases with cancer, biological activity of a product, presence or absence of immunosuppressive action, *in vitro* data, etc. In those cases where the product is biologically active and non-immunogenic in rodents and other studies have not provided sufficient information to allow an assessment of carcinogenic potential, then the utility of a single rodent species should be considered. Careful consideration should be given to the selection of doses. The use of a combination of pharmacokinetic and pharmacodynamic endpoints with consideration of comparative receptor characteristics and intended human exposures represents the most scientifically based approach for defining the appropriate doses. The rationale for the selection of doses should be provided.

Points to consider on the non-clinical assessment of the carcinogenic potential of insulin analogues have been published (CPMP, 2001).

Local tolerance studies

Local tolerance should be evaluated using the dosage form to be clinically used or similar dosage form if appropriate. As described in the guideline, local tolerance does not mean eye- or skin-irritation safety studies for personnel engaged in manufacturing of CSC. It means there is irritable response at the injection site. In some cases, the potential adverse effects of the product can be evaluated in single- or repeated-dose toxicity studies, thus obviating the need for separate local tolerance studies.

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REFERENCES

CPMP/372/01 (2001): Points to consider on the non-clinical assessment of the carcinogenic potential of insulin analogues. www.emea.eu.int.

CPMP/SWP/2600/01 (2002): Points to consider on the need for assessment of reproduction toxicity of human insulin analogues. www.emea.eu.int.

Ministry of Health and Welfare (2000): Preclinical safety evaluation of biotechnology-derived pharmaceuticals. Notification No. 326.

Pharmaceutical Non-clinical Investigation Group (2002): "Points to consider" regarding safety assessment of biotechnology-derived pharmaceuticals in non-clinical studies. In Handbook on non-clinical guidelines for pharmaceuticals 2002, pp.83-94, Yakujinipposya, Tokyo.

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Mechanism of Benzene-Induced Hematotoxicity and Leukemogenicity: Current Review with Implication of Microarray Analyses

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ABSTRACT

Benzene is a potent human leukemogen but the mechanism underlying benzene-induced leukemia remains an enigma due to a number of questions regarding the requirement of extraordinarily long exposure, a relatively low incidence of leukemia for genotoxicity of metabolites and a narrow dose range for leukemogenicity over marrow aplasia (overdoses tend to result in marrow aplasia). Moreover, there were previous controversies as to whether the cell cycle is upregulated or suppressed by the benzene exposure. Subsequently, it was found that the cell cycle is suppressed, but how leukemia develops under such suppression of hemopoiesis remains to be clarified. These questions were fortunately resolved with much effort. Benzene exposure was found to induce the expression of p21, an interlocking counterdevice for cell cycle: due to *p53* upregulation, thereby inducing the immediate suppression of the kinetics of hemopoietic progenitors followed by the prominent suppression of hemopoiesis. Intermittent benzene exposure (i.e., cessation of exposure during weekends, for example) allowed an immediate recovery from marrow suppression after terminating exposure, which induced continuous oscillatory changes in marrow hemopoiesis. Benzene-induced leukemia was chiefly due to such an oscillatory change in hemopoiesis, which epigenetically developed leukemia more than 1 year later. The mechanisms of benzene-induced leukemogenicity seem to differ between wild-type mice and mice lacking *p53*. For *p53* knockout mice, DNA damage such as weak mutagenicity or chromosomal damage was retained, and such damage induced consequent activation of proto-oncogenes and related genes, which led cells to undergo further neoplastic changes. In contrast, for wild-type mice carrying the *p53* gene, a marked oscillatory change in the cell cycle of the stem cell compartment seems to be important. Compatible and discriminative gene expression profiling between the *p53* knockout mice and wild-type mice was observed after benzene exposure by microarray analyses.

Keywords. Benzene; hematotoxicity; leukemogenicity; gene chip array; BUUV method; *p53*-KO; AhR-KO; hemopoietic progenitor cells.

INTRODUCTION

The mechanism of benzene-induced leukemia had long been an enigma until recently, when the unique cell kinetics of stem/progenitor cells during benzene exposure was elucidated. Leukemia induction by benzene inhalation was first reported in 1897, when Le Noire described multiple cases of leukemia among Parisian cobblers (Le Noir and Claude, 1897). However, the experimental induction of leukemia by benzene exposure was first reported about 20 years ago by Snyder et al. (1980) and our group (Cronkite et al., 1984, 1989). Recently, we demonstrated marked oscillatory changes in peripheral blood and bone marrow (BM)[1] cellularities during and following benzene inhalation, preceding the development of leukemia by about 1 year (Hirabayashi et al., 1998; Kawasaki et al., 2001; Yoon et al., 2001).

Benzene-induced leukemia is unique in that it has been associated only with a weak mutagenicity in the benzene metabolites, phenol and hydroquinone. Another interesting observation is the controversial experimental data concerning the level of actively cycling hematopoietic cells following benzene exposure. While all researchers observed a decrease in peripheral blood and BM cellularities, some observed a suppression of the cell cycle of BM, as measured by tritiated thymidine incorporation (Moeschlin and Speck, 1967), whereas others observed a marked increase in the number of cycling stem/progenitor cells in BM and peripheral blood (Table 1). Careful analysis of these apparently conflicting data revealed an enhancement of the cell cycle occurring at least 2 hours after the termination of benzene exposure. Thus, the higher tritiated thymidine incorporation documented by Cronkite et al. (1982) 18 hours after the termination of benzene exposure probably reflects a recovery phase. Based on these findings, we conducted a series of studies since 1997 to elucidate the leukemogenic effect of benzene in wild-type mice.

The *p53*-knockout (KO) mouse (Tsukada et al., 1993) showed further unique characteristics of benzene-induced leukemia. Using *p53*-KO mouse, we confirmed that benzene has a moderate genotoxic effect, as measured by the micronucleus test performed 4 weeks after the initiation of benzene inhalation (Kawasaki et al., 2001; Li et al., 2003). Moreover, *p53*-deficient mice manifest increased susceptibility to

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[1]Abbreviations: BM, bone marrow; KO, knockout; UV, ultraviolet; BUUV, incorporation of bromodeoxyuridine followed by ultraviolet-light cytocide to evaluate the hemopoietic stem/progenitor cell kinetics in vivo; AhR, aryl hydrocarbon receptor; $AhR^{+/+}$, AhR wild-type; $AhR^{+/-}$, AhR heterozygous-deficient; $AhR^{-/-}$, AhR homozygous-deficient; CFU-GM, granulo-macrophage colony forming unit; CYP, cytochrome P450; FGF, fibroblast growth factor; TGF, tumor growth factor; I.V., intravenous; I.P., intraperitoneal; aft, after; dur, during; expos, exposure.

TABLE 1.—Summary of the results the hemopoietic stem/progenitor cell kinetics during and after benzene exposure by tritiated thymidine (^{3}H-TdR) cytoci assay.

		Cellularity		Evaluation cell and assay methods					
				BM cells			CFU		
Year	Reference	Blood	BM	Kinetics	Labeling*,1	Label point	Kinetics	Labeling	Label point
1967	Moeschlin and Speck	↓	↓	↓	In vivo	At pancytopenia	—	—	—
1979	Irons et al.	↓	↓	↑	In vivo[2]	6 days aft. expos-IP	—	—	—
1982	Cronkite et al.	↓	↓	—	—	—	↑	In vitro	18 h aft. expos.
1998	Lee et al.	↓	↓	↓	In vivo[3]	30 min aft. single IP	—	—	—
		↓	↓	↓	In vitro	Dur. and aft. expos.	—	—	—
1997	Farris et al.	↓	↓	→↓	In vivo[4]	Soon aft. expos.	↑	In vitro	2 h aft. expos.

1. ^{3}H-TdR was injected intravenous (IV) at in vivo labeling except indications.
2. ^{3}H-TdR was injected intraperitoneal (IP) 6 days after cessation of benzene.
3. Benzene was treated single IP, and ^{3}H-TdR label was starting 30 minutes after benzene treatment.
4. Instead of ^{3}H-TdR, BrdUrd was used for assay.

benzene-induced leukemogenicity (Kawasaki et al., 2001). Similar findings with regard to increased leukemogenicity following benzene exposure have been documented by French et al. of the National Institute of Environmental Health Sciences (French et al., 2001). Contrary to the result in *p53*-KO mice, benzene-induced leukemia had not been detected in earlier studies in wild-type mice because its manifestations had been masked either by pancytopenia due to severe myelosuppression or by the use of a benzene dose too low to induce pancytopenia or leukemia (Kawasaki et al., 2001). Aryl hydrocarbon receptor (AhR)-KO mice (Mimura et al., 1997) also elucidated the characteristic underlying mechanism of benzene-induced hematotoxicity (Yoon et al., 2002).

In the mechanism underlying benzene toxicity in BM tissue analyzed using a microarray system, various signaling pathways have been suggested to be implicated including cell cycle regulation, DNA-damage/repair-related genes, oxidative-stress-related genes, growth-factor-related genes, oncogenes, and hemopoiesis-related genes in general (Yoon et al., 2003).

OSCILLATORY CHANGES IN BONE MARROW CELLULARITY IN WILD-TYPE MICE

BM cellularity decreases markedly during benzene inhalation but recovers rapidly following the termination of benzene exposure (Yoon et al., 2001). The oscillatory nature of the resultant curve is comparable to the response reported by Cronkite et al. (1984, 1989), and suggests that benzene does not only induce BM cell suppression but also activates cell-cycle-regulating genes, resulting in compensatory myelopoiesis.

We used the BUUV (bromodeoxyuridine + UV exposure) method to study stem/progenitor cell kinetics during or after benzene exposure (Hirabayashi et al., 1998; Yoon et al., 2001). Using this method, we were able to measure the labeling rate, cycling fraction of clonogenic progenitor cells, and other cell cycle parameters. Interestingly, the cycling fraction of stem/progenitor cells was found not to turn into active hematopoiesis but to remain low during benzene inhalation. Furthermore, rapid recovery was observed after benzene inhalation was terminated (Figure 1). However, although the exact mechanism of this phenomenon is not yet known, we found the evidence that the cycling fraction depression may be mediated in part by the suppression of stem/progenitor cell cycling per se, owing to the *p53*-dependent upregulation of p21 (Yoon et al., 2001). Thus, the mechanism of benzene-induced leukemia in the wild-type mice may be due to continuous oscillatory changes in hemopoiesis during and after the benzene exposure, which leads to genetic instability followed by the consequent epigenetic leukemogenicity.

p53-DEFICIENT MICE DEVELOP LEUKEMIA BY DIFFERENT MECHANISMS

Leukemogenicity induced in *p53*-KO mice, because of the lack of the *p53* gene, results in the noninduction of p21 expression even during the benzene exposure, with subsequent insufficient DNA repair and accumulation of DNA damage. These pathways are shown in Figure 2 for benzene-induced possible toxicological changes in both wild-type and *p53*-KO mice. In *p53*-KO mice, cell cycle suppression, DNA repair, and apoptosis of damaged cells, which, in general, occur in the wild-type mice after benzene exposure, are all suppressed. This is much more likely genotoxic leukemogenesis, in which reactive oxygen species, dysfunction of topoisomerase, and covalent binding of adduct formation to DNA,

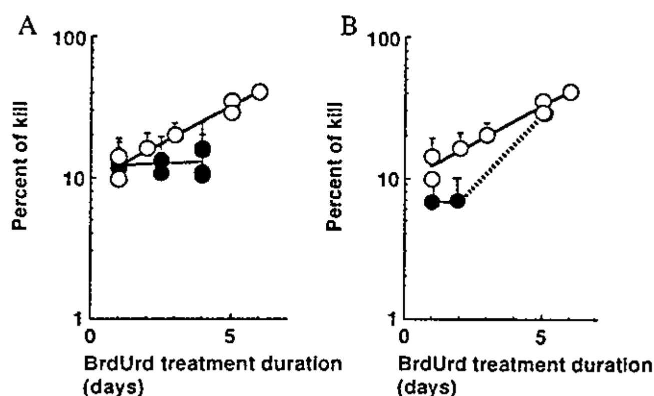

FIGURE 1.—Hemopoietic progenitor cell (CFU-GM) kinetics during (A) and after (B) benzene inhalation. Open circle: sham; Closed circle: during or after inhalation of 300 ppm benzene, 6 h/day, 5 days/week ×2 weeks. (A) For the benzene-treated group, all the mice were sacrificed just after the 5th day of the 2nd week of benzene-inhalation. The osmotic minipump filled with BrdUrd was implanted into donor mice day(s) before sacrificing as indicated on the abscissa. (B) For the benzene-treated group, the BrdUrd-pump was implanted into donor mice after the 5th day of the 2nd week of benzene-inhalation and sacrificed on the day as indicated on the abscissa. Each point represents at least 2 mice as a donor for the CFU-GM assay, and colony assays were performed in triplicate.

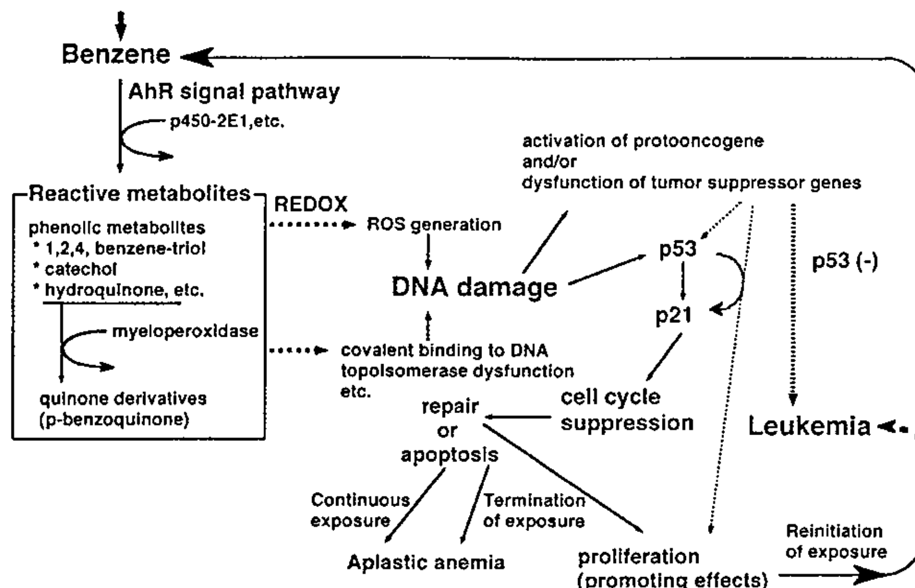

FIGURE 2.—Benzene metabolism and possible mechanism of benzene-induced leukemogenesis.

all synergistically participate in further leukemogenic development without repairing the system (see Figure 2). Thus, leukemogenicity seems to be clearly different between the mice carrying wild-type *p53* and the mice lacking *p53* (Yoon et al., 2001; Hirabayashi et al., 2002).

ARYLHYDROCARBON-RECEPTOR-MEDIATED BENZENE METABOLISM

We investigated the involvement of the aryl hydrocarbon receptor (AhR), a ligand-activated basic helix-loop-helix transcription factor, in hematotoxicity using AhR wild-type ($AhR^{+/+}$), heterozygous-KO ($AhR^{+/-}$) and homozygous-KO ($AhR^{-/-}$) male mice (Mimura et al., 1997; Yoon et al., 2002). Following a 2-week inhalation of benzene at 300 ppm, we evaluated the changes in cellularity of the peripheral blood and BM, and the levels of granulocyte-macrophage colony-forming units (CFU-GM) in the BM (Figure 3). The expression of the cyclin-dependent kinase inhibitor, p21, in BM cells and cytochrome P450 (CYP) 2E1 in hepatic tissues were evaluated by Western blot analysis after benzene exposure. Our results clearly showed that $AhR^{-/-}$ mice are much more resistant to the benzene-induced hematotoxicity than $AhR^{+/+}$ wild-type mice. No changes in p21 expression level by BM cells were detected in $AhR^{-/-}$ mice, whereas a marked upregulation of p21 expression by BM cells was observed in $AhR^{+/+}$ mice. This finding is a further proof of the resistance of $AhR^{-/-}$ mice to benzene-induced hematotoxicity. The benzene resistance of $AhR^{-/-}$ mice was abrogated by exposure to a combination of 2 major metabolites, phenol and hydroquinone, strongly supporting the notion that AhR participates in benzene metabolism. CYP species involved in such metabolism are under investigation. The results obtained imply that pollutants that react with AhR confer marked susceptibility to benzene-induced hematotoxicity.

IMPLICATION OF MICROARRAY ANALYSIS

In the mechanism underlying benzene toxicity in BM tissue, various signaling pathways have been suggested to be implicated including metabolism, genotoxicity, cell cycle regulation, and apoptosis (Table 2). Our microarray analysis

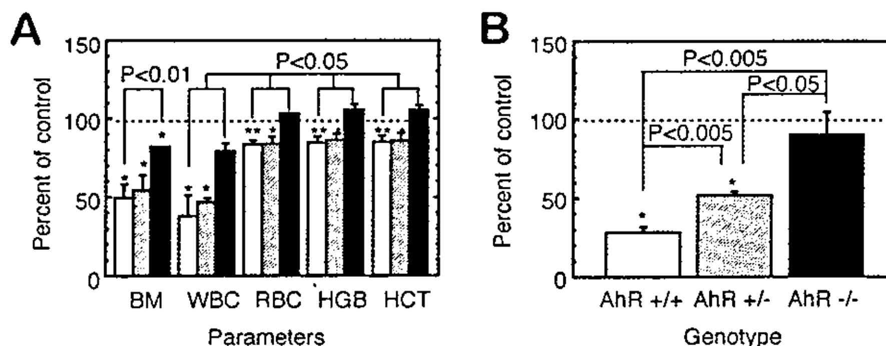

FIGURE 3.—Changes in peripheral blood parameters and BM cellularity (A) and CFU-GM per 2 femurs (B) in the AhR wild-type ($AhR^{+/+}$:open bar), heterozygous-KO ($AhR^{+/-}$:shaded bar) and homozygous-KO mice ($AhR^{-/-}$:closed bar) exposed to 300 ppm benzene for 2 weeks. The mean BM cellularities for the $AhR^{+/+}$, $AhR^{+/-}$, and $AhR^{-/-}$ mice were 4.8, 5.6, and 4.8 × 10^7/2 femurs, respectively, and the mean numbers of CFU-GM's per 8 × 10^4 BM cells was 79, 78, and 72, respectively. *, **: Significantly different from each corresponding control group at $p < 0.05$ and $p < 0.01$, respectively.