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Investigation of hyaluronan function in the mouse through targeted mutagenesis

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It has become increasingly apparent that the high molecular mass glycosaminoglycan, hyaluronan (HA), is required for many morphogenetic processes during vertebrate development. This renewed understanding of the various developmental roles for HA, has come about largely through the advent of gene targeting approaches in the mouse. To date, mutations have been engineered in the enzymes responsible for biosynthesis and degradation and for those proteins that bind to HA within the extracellular matrix and at the cell surface. Collectively, the phenotypes resulting from these mutations demonstrate that HA is critical for normal mammalian embryogenesis and for various processes in postnatal and adult life (Table 1). In this article we will review our progress in understanding the biological functions for HA through targeted mutagenesis of the HA synthase 2 (*Has2*) and 3 (*Has3*) genes. Data that has been obtained from a conventional targeted disruption of the *Has2* gene, is presented in an accompanying review by Camenisch and McDonald. More specifically, in this review we will provide an overview of the conditional gene targeting strategy being used to create tissue-specific deficiencies in Has2 function, along with our progress in understanding the role for Has3-dependent HA biosynthesis.
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HA is unique among the glycosaminoglycans, in that it is synthesized and released from the cell as a linear, unmodified polymer that is not covalently attached to a polypeptide backbone. HA is synthesized at the plasma membrane by any one of three HA synthases [1,2], integral plasma membrane proteins that draw off a cytosolic pool of activated UDP-sugar precursors as substrate. Heterologous expression of any one HA synthase in a given cell line, converts that line into a factory for HA biosynthesis [2,3]. A preliminary expression screen indicated differential expression of *Has* genes during mouse embryonic development and in the adult, suggesting that promoters have diverged [2]. In addition to differences in the regulation of gene expression, it is clear that each HA synthase has characteristic differences in enzymatic activity, primarily relating to V_{max} and relative binding affinities for the two UDP-sugar substrates [2,3]. Thus, it is still formally possible that while each HA synthase catalyzes the biosynthesis of HA, the three enzymes may not be able to replace each other functionally during all aspects of development or adult life.

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To investigate the biological functions for the three HA synthases and their biosynthetic product, we have used conventional targeted mutagenesis. While the targeted inactivation of *Has1* (Itano, Spicer, Kimata and McDonald, unpublished data) and *Has3* (Spicer and McDonald, unpublished data) produced viable homozygous null animals, mice homozygous for the *Has2* null allele were not obtained; Has2 deficiency resulted in an embryonic lethal phenotype at E10.5 [4], which is described in more detail by Camenisch and McDonald in an accompanying review. Thus, HA is indispensable for normal mouse embryogenesis.

It is perhaps not too surprising that loss of Has2 function resulted in embryonic lethality, given its normal pattern of expression. Has2 is expressed most highly in the mid-gestation mouse embryo, where it correlates with sites previously described as being rich in HA. These include the endocardial cushions of the developing heart, craniofacial mesenchyme, precartilage areas and neural crest (Figure 1A). In contrast, Has1 is expressed only during gastrulation (E7.5–E8.5) in a more general pattern (data not shown), and Has3 is expressed only by the mesenchymal component of developing teeth and hair (Figure 1B–F).

As a large, space-filling, organizer of the extracellular matrix, one of HA's primary functions may be to regulate how cells interact with their neighbors. A large organized HA-dependent

Table 1. Phenotypes from gene knockout mutations relating to hyaluronan synthesis and function

Gene name	*Mutation*	*Phenotype*	*Conclusion*
AGGRECAN	cmd[1] (Spontaneous)	Postnatal lethality due to severe skeletal dysplasia	Aggrecan required for normal skeletogenesis
Link Protein[2]			
CD44	Targeted null[3]	Minor phenotypes relating to lymphocyte trafficking and tumorigenicity	CD44 not absolutely required for normal development.
VERSICAN (Cspg2)	Transgenic Insertion[4]	E10.5 embryonic lethal	Versican required for normal endocardial cushion formation
Has2	Targeted null[5]	E10.5 embryonic lethal	Has2 and HA required for endocardial cushion formation, epithelial-to-mesenchymal transformation, vasculogenesis and neural crest survival.
Neurocan	Targeted null[6]	Viable and fertile; mild defects in hippocampal long-term potentiation	Neurocan is not required for normal brain development.
Has3	Targeted null[7]	Viable and fertile; tooth, fertility and skin phenotypes	Partial redundancy between Has2 and Has3.

[1][18]; [2][19]; [3][20]; [4][21]; [5][4]; [6][22]; [7]Spicer et al., unpublished data.

extracellular matrix may effectively inhibit cell-cell adhesion. Accordingly, if such a matrix is endocytosed or degraded, cell-cell contact may be achieved. This is essentially the process underlying condensation, which occurs for instance during chondrogenesis and hair follicle development. HA may play a key role in the regulation of the timing of condensation and, hence, the number of cells that may be ultimately present within a given condensation. Has2 is expressed in cartilage prior to condensation, then is down regulated in condensed, proliferating chondrocytes (data not shown). Has3 is the dominant HA synthase expressed in developing hair follicles (Figure 1B and C), although Has2 is also expressed at lower levels.

Based upon the observation that Has2 and Has3 are the most closely related HA synthases, at the amino acid and gene structure levels [2], we investigated the phenotypes associated with compound loss of both Has3 and Has2 function. Animals doubly heterozygous for both Has2 and Has3 (*Has2*+/−, *Has3*+/−) were viable, healthy and fertile. However, a significant fraction of animals homozygous for Has3 and heterozygous for Has2 (*Has2*+/−, *Has3*−/−) presented with obvious malocclusions of the teeth before or shortly after the time of weaning (data not shown). These animals were obtained at a frequency of 14% on an outbred genetic background, suggesting that at least one other major gene is involved in modifying the tooth phenotype. As both Has2 and Has3 are expressed by the tooth-forming cephalic neural crest cells, it is highly likely that there is partial redundancy of function in this cell population, with respect to the source of HA. It is also likely that the malocclusions observed in these animals result from a defect that is intrinsic to the tooth-forming crest. This may be related to pathfinding of this cell population, or to proliferation or differentiation. All of these aspects are currently under investigation. The major modifier gene locus is also being sought. In addition to the tooth phenotype observed in compound *Has2*+/−, *Has3*−/− animals, we have observed reduced fertility and a variety of skin phenotypes, mostly related to excessive scratching. Has2 and Has3 are both expressed within the ovary [2,5,6] with Has2 being specifically induced during cumulus cell expansion [5,6]. Litter sizes are reduced in *Has2*+/−, *Has3*−/− animals and numbers of litters per year are also reduced. Has3 is highly expressed within the epidermis [7], and we believe that the skin phenotypes may be simply explained by excessively dry skin, due to loss of HA-associated water. A marked reduction in the amount of HA within the epidermis, may affect terminal differentiation and loss of dead skin cells. Again, we are investigating the basis for these phenotypes. Overall, results from our conventional HA synthase gene knockouts have shown that Has2 is the primary HA synthase within the developing embryo and is necessary and sufficient for normal embryogenesis. In addition, Has2 and Has3 are partially redundant within certain cell populations.

After E10.5, Has2 expression is maintained within the cushion tissue of the heart, but also becomes very obvious within the developing embryonic skeleton, where it is expressed by the precondensing cartilage, early cavitating joints and hypertrophic zones (data not shown). In the adult, Has2 is the target of induction by many growth factors and cytokines [6,8–11]. Thus, the ability to manipulate the Has2-dependent HA biosynthetic machinery in the embryo after E10.5 and in the adult would provide us with a wealth of new data regarding the various biological roles for HA. To achieve this goal, we have spent some time designing a vector system to apply allelogenic gene targeting [12] to the mouse *Has2* gene

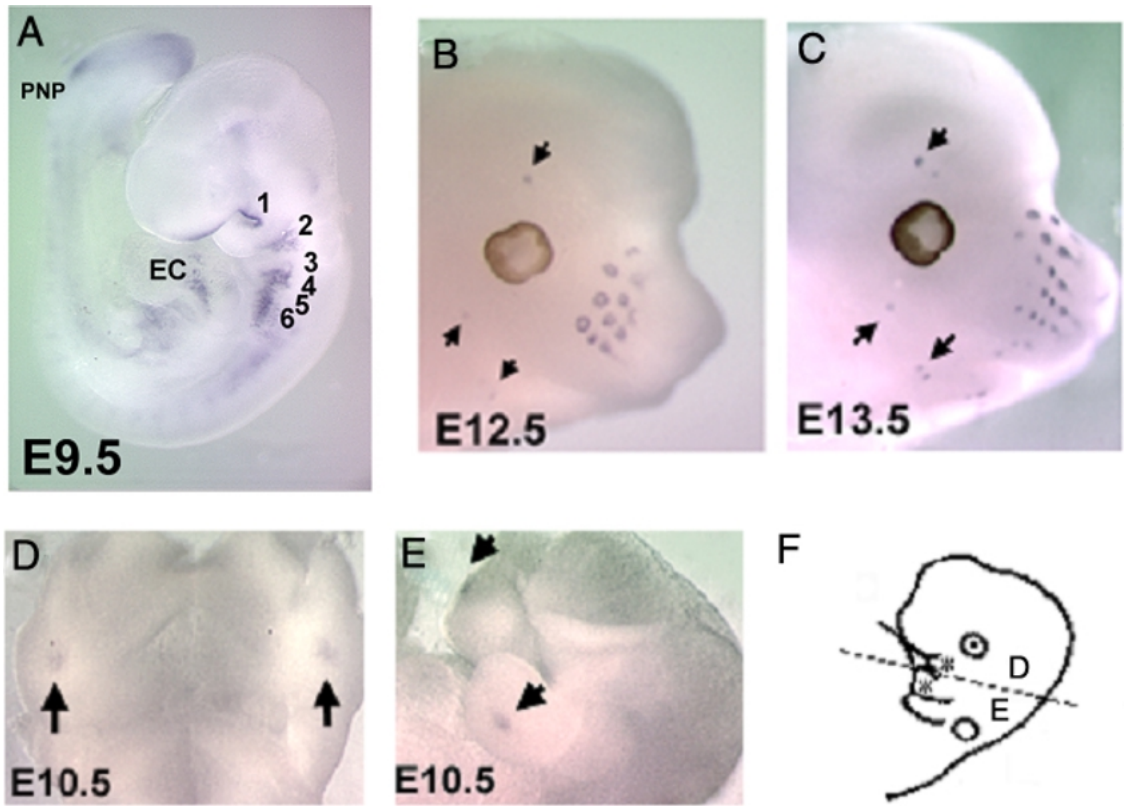

Figure 1. Expression of Has2 and Has3 in the Developing Mouse Embryo. Whole-mount *in situ* hybridization was carried out essentially using published procedures [23] with digoxigenin-labeled antisense RNA probes for mouse Has2 or mouse Has3 or the equivalent sense probes. Colorimetric detection was employed. (A) Has2 expression was observed in neural crest cell populations and the endocardial cushions at E9.5. Branchial arches 1–6 are numbered. (B) and (C) Has3 is expressed within developing whisker and hair follicles in a transient fashion. (D)–(F) Has3 is expressed by tooth-forming cephalic neural crest cells within the first branchial arch at E10.5. Arrows indicate the areas of tooth-forming cephalic neural crest cells that are positive for Has3 expression. (F) Cartoon depiction of an E10.5 mouse embryo. The dashed line indicates the plane at which the embryos were cut to view the positive *in situ* signal in the maxillary (D) and mandibular (E) components of the first branchial arch. EC, endocardial cushions; PNP, posterior neuropore.

locus (Figure 2). Using this approach, recognition targets for two site-specific recombinases are employed and incorporated into the targeted allele, by homologous recombination. The first targeted allele is often hypomorphic, or leaky, resulting in a partial loss of function. This type of allele is often extremely informative as it allows creation of lines with substantially reduced gene product function [12–15]. Most conventional gene knockouts do not present with a phenotype in the heterozygous state. This would suggest that most developmental processes could proceed normally despite a significant reduction in the amount of any given gene product. We assume, for sake of argument, that most heterozygotes will produce roughly 50% of the wild-type level for a given gene knockout. The results obtained from the creation of hypomorphic alleles suggest that phenotypes are revealed when a given gene product is reduced to 5 or 10% of normal levels [12–15]. Varying amounts of the gene product can be achieved by making the hypomorphic allele homozygous or by combining it with a null allele (Figure 2).

The strategy we have used to create our *Has2* conditional allele is outlined in Figure 2. A selectable marker cassette (PGKneo) has been inserted into intron 3, and is flanked by target sites for Flp recombinase. Recognition sites for Cre recombinase have been placed on either side of exon 3. Exon 3 is the smallest *Has2* exon, encoding 34 amino acids of the enzyme, including a critical residue thought to play a role in coordinating the metal cation, which is required for enzyme activity [2,16]. Deletion of this exon would thus result in the creation of a functionally null *Has2* allele. We chose to flank exon 3 with loxP sites and to insert the neo cassette into intron 3, as we believe that this has the highest probability of generating a hypomorphic allele. In addition, exonic sequences for a gene that is transcribed from the opposite strand of *Has2* are located within intron 1 of *Has2*, along with enhancer/regulatory sequences for *Has2* and this new gene (Spicer et al., unpublished results). Thus, we were hesitant to place a selectable marker cassette or loxP sites into or around exon 2.

In the course of generating targeted embryonic stem cell clones using this strategy, two different targeted clones were obtained (Figure 2 and data not shown). The first clone incorporated only a single loxP site into the targeted allele, creating

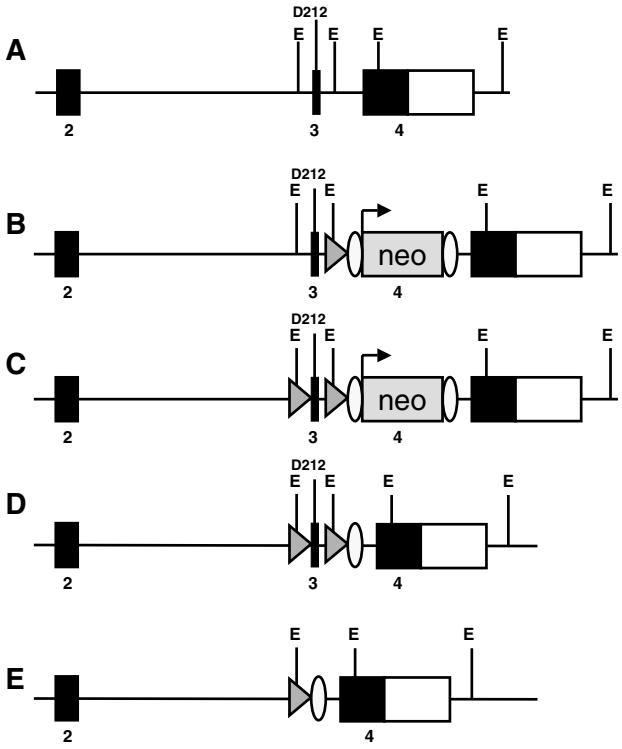

Figure 2. Strategy for Allelogenic Type Conditional Gene Targeting of the *Has2* Gene. (A) The previously described structure of the mouse *Has2* gene. Homologous recombination should introduce two loxP sites and a FRT—flanked PGKneo cassette into the *Has2* gene locus. (B) The predicted structure of a correctly targeted clone. (C) If homologous recombination occurs between the two loxP sites, the targeted allele will retain only a single loxP site. Both targeted alleles (B and C) are expected to be hypomorphic with respect to Has2 function. Furthermore, both targeted *Has2* alleles can be manipulated in the ES cells or *in vivo* (in the mouse) by expression of Flp recombinase [12,17]. (D) In the presence of Flp, the PGKneo cassette is specifically deleted, leaving behind a single FRT site. This is expected to revert the hypomorphic *Has2* alleles to functionally wild-type alleles. (E) The allele containing two loxP sites can also be manipulated *in vitro* (embryonic stem cells) or *in vivo*, this time using Cre recombinase. Cre recombinase will act to specifically delete exon 3, creating a functionally null *Has2* allele as explained in the text. Cre recombinase is typically expressed under the control of a spatially and temporally regulated, or inducible promoter. Multiple transgenic mouse lines have now been reported in which Cre recombinase is regulated in this manner. Filled black boxes, Has2 open-reading frame; open box, Has2 3′ untranslated region; filled gray boxes, PGKneo cassette; filled gray arrowheads, loxP sites; open ovals, FRT sites; numbers indicate exons for Has2; D212 indicates the relative position of the codon encoding the critical aspartate (D) residue at position 212 within the predicted polypeptide chain (E) EcoRI sites.

a predicted hypomorphic allele, but one that cannot be recombined using Cre recombinase. This clone resulted from a homologous recombination event that was localized between the two loxP sites within the targeting vector and one homologous chromosomal copy of the *Has2* gene, resulting in loss of one loxP site from the recombined allele. The second targeted clone incorporated both loxP sites into the recombined allele. Targeted clones were obtained at a frequency of 1 in 200 antibiotic resistant clones; a frequency similar to that observed for our conventional gene knockout [4].

The first targeted clone that was obtained will be useful for creating a hypomorphic mouse line, as described above. In addition, we are using this embryonic stem cell and mouse line to permit high fidelity targeting of transgenes to the *Has2* gene locus, using coelectroporation or coinjection of recombinant Cre enzyme along with the transgene DNA containing a single loxP site. Trans-recombination between the single loxP site within the targeted *Has2* allele, and the loxP site carried alongside the transgene DNA, results in a higher percentage of transgene insertion events taking place at the *Has2* gene locus through Cre-catalyzed homologous recombination. Using this strategy, we are creating chimeric *Has2* alleles, in which the sequences encoded by exon 4 of *Has2* are replaced by the equivalent sequences from Has3 or Has1. It is these sequences that appear to specify the differences in enzymatic activity that have been previously described for Has1, Has2 and Has3 [2,3]. These experiments are ongoing and will allow us to investigate the functional relationships between *Has* gene products *in vivo*.

The second targeted clone will represent the most useful strategy for investigating HA function in many developmental processes and in normal adult physiology. First, the hypomorphic allele is generated. Next, the hypomorphic allele is reverted back to an essentially wild-type allele, using Flp recombinase *in vivo* [17]. Once animals with the desired genotype have been derived, crosses are established in which offspring are predicted to have genotypes where Cre-mediated loss of Has2 function will result in cell populations or tissues with essentially no ability to synthesize HA. By performing all of these crosses on a *Has3*-null background, we will avoid the possibility of rescue/redundancy of *Has2* through expression of Has3. Targeted clones and initial mouse lines have now been derived. These will provide us and other investigators with an invaluable tool with which we will investigate and uncover the true biological functions for this fascinating molecule.

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