Pinpointing the cause of the difference between Hras and Kras

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Abstract
Ras proteins are small GTPases that play a key role in cell proliferation and differentiation. Ras is essential for controlled cell growth, however, overexpression of Ras can create uncontrolled cell growth and lead to cancer. There are three Ras genes Hras, Nras, and Kras. The different differences between Ras genes can hold clues to the specific mechanisms of how cancer works and differs. On a genetic level, Kras and Hras are quite similar except for a couple of locations, the biggest difference between the two is located at the C-terminal the tail and is called the Hypervariable Region (HVR). Previous experiments have shown that in fact swapping the two tail sections did not alter the phenotype of the given Ras gene. Therefore, the difference must lie somewhere else on the gene. By locating the areas that differentiate the two Ras', a great deal of information about Ras and cancer could be answered. The different genes can help provide insight on what makes Hras so much more lethal than Kras and provide information regarding why differentiation is blocked in Hras induced AML but not blocked CMML.

Introduction
Analysis of the human genome and other animals’ genomic sequences have helped to propel and vastly improve our understanding of how cancer functions. In order to combat cancer, there first must be an understanding of how cancer takes forms and
develops. Cancer is caused by abnormal cell proliferation, lack of apoptosis, and sometimes differentiation. 30% of cancers are caused by the mutation of the Ras genes. Of the 30% of cancers caused by the mutated Ras, about 20-30% are considered acute myeloid leukemia (AML) and about 50-70% are chronic myelomonocytic leukemia (CMML). Ras proteins are small GTPases that act as switches to help achieve much of the regulation that is needed to help maintain the cell.

Ras still has many mysteries shrouding its exact function. Ras is so complicated and difficult to understand because of the amount of regulators that can affect Ras and the amount of downstream effectors it can activate. Ras has three major pathways that it affects downstream when it encounters a mutation; commonly represented in experiments as 37G (glycine), 35S (serine), 40C (cysteine). The 35S, starts with Ras activating the Raf by promoting Raf’s translocation to the plasma membrane where it waits to be fully activated. Raf phosphorylates and then activates MEK’s intermediary kinases, then the MEK phosphorylates the ERK intermediary kinases. The activated Erk then moves into the nucleus where it then phosphorylates and activates different transcription factors such as Elk1.

Another major pathway is going through 40C. Ras stimulates the lipid kinase activity of PI3K, which phosphorylates PtdIns (4, 5)p2. This then interacts with the next two downstream effectors, Akt/PKB and Rac GDP-GTP. Akt/PKB phosphorylates multiple effectors which all contribute to anti-apoptosis in the cell, either by inhibiting an inhibitor for anti-apoptotic protein or by stimulating the expression of anti-apoptotic genes, NF-kB. The RacGDP-GTP also stimulates the expression of anti-apoptotic
genes by stimulating the release of NF-kB\textsuperscript{[vi]}. The increased anti-apoptotic genes might play a big role in the oncogenic form of Ras, since an overabundance of this path would most likely create an imbalance and possibly get out of control, leading to cancer.

When Ras binds to RalGDS, it activates GDP-GTP exchange factors while activating Ras-related small GTPases, which in turn inhibits Cdc42 and Rac GTPases. This is the same Rac that is used to promote the release of NF-kB, which stimulates the expression of anti-apoptotic genes. Ral also helps to activate phospholipase D1 that in turn catalyzes the conversion of phosphatidyicholines to phosphatidic acid\textsuperscript{[vii]}. These are three of the better known Ras pathways, however just within these pathways there are many questions left to be understood, such as the actual involvement some of the effectors have on the functioning of Ras.

Not only does Ras have complicated molecular pathways; there are also multiple types of Ras proteins, Hras, Nras, Kras 4A, and Kras 4B\textsuperscript{[viii]}. These different Ras proteins are linked with different forms of cancer. Each Ras gene has been linked to different types of tumors. Kras is linked more often to pancreatic, lung and colon cancers; Nras is associated with hepatocellular carcinoma; Hras is linked to papillary thyroid cancer\textsuperscript{[ix]}. Also in myeloid malignancies it has been found that Nras mutations also tend to appear more frequently than Kras while Hras mutation occur much less frequently than Kras\textsuperscript{x}.

Ras was thought to have almost identical functions between the four type of Ras proteins, most likely due to their very similar genomic sequences. However recent studies have revealed differences in the functions of each of the proteins\textsuperscript{xii}. Kras has been seen to have high levels of embryonic fatality in knockout mice, while Hras and
Nras do not share this same trait. Furthermore it was shown that Hras always induced an AML-like disease, while Kras induced a CMML-like disease. Nras however produced both AML-like and CMML-like diseases in the mice. Aside from the types of tumors they can induce, a test on mice showed that all three different forms of Ras can induce myeloid leukemias in the mice however they do so at different rates.

The structural differences between the different forms of Ras have been the focus of my experiments. Since it has been tested that Hras always induces an AML-like disease and Kras induces a CMML-like disease. The big difference between AML and CMML is that AML blocks differentiation of cells while CMML does not. The prevention of differentiation is what makes AML so much more lethal. It was important to know what causes the blocked differentiation, so the location of the genes that caused the difference had to be found. An experiment was done to test for the effects of swapping the C-terminal (hypervariable region) of Hras and Kras, since that region has the most gene differences in the two Ras gene sequences. If the hypervariable region contained the essential gene needed to make the difference, it would express the phenotype of the tail Ras. If neither Hras or Kras exhibited the other one's phenotype then the amino acid needed to trigger the differentiation block, must be located somewhere else. The next step would be to analyze a region of amino acids that is not as differentiated as the hypervariable region but still has several differences, in my case part of the G domains of H-ras and K-ras. Then by swapping G domains of Hras and Kras we can see if there are any changes in the expression of phenotype by the mice. This will help us narrow in on what regions actually make the different forms of Ras different from each other.
Materials and Methods

Data Constructs


Process for the constructs will be detailed in the data section.

Cell Culture and Retrovirus Production

NIH3T3 fibroblast cells were grown in DMEM (Dulbecco’s modified Eagle medium) supplemented with 10% DBS (donor bovine serum) and 1x penicillin/streptomycin.

BOSC23 cells were grown in DMEM supplemented with 10% FBS (fetal bovine serum) and 1x penicillin/streptomycin. Both NIH3T3 and BOSC23 cells were cultured in 100mm plates in humidified incubators with 5% CO$_2$ at 37$^\circ$C.

BOSC23 cells were used as the retroviral packing cell line, and are used for the transfection. The BOSC23 cells were split at $1.5 \times 10^6$ cells per 60mm plates 18 hours before the transfection. The BOSC23 cells are transfected with the MSCV vectors/constructs. The cells were transfected using calcium chloride and 2X HBS to help form the DNA precipitate. The cells were incubated for 10 hours in the incubator, then the plates were given new fresh media and left in the incubator for another 48 hours.
The viral supernatant is then collected and tested with infected NIH3T3 cells for GFP expression. The viral titer (calculated in TUs, transducing units) is calculated by multiplying the number of NIH3T3 cells with GFP expression and the total number of cells in the plate at the time of infection. This viral titration allows for a more accurate representation of the potency of each different MSCV constructs by equalizing GFP expression.

**Bone Marrow Transduction**

Donor Balb/c mice were injected with 25mg/mL of 5-FU (5-fluorouracil) via the lateral tail vein. The bone marrow cells were then harvested from the mice's femurs and tibias. The cells are then isolated and infected with the previously harvested retrovirus. The infected bone marrow cells are then injected into lethally irradiated recipient Balb/c mice via the lateral tail vein.

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**Data**

![Amino Acid Sequence Diagram](image)

*Figure 1. Amino Acid sequences for Hras, Nras, and Kras.*
MSCV-ires-GFP-KrasD12 with HrasD12 mutations at G1 construct (KwH1) and MSCV-ires-GFP-KrasD12 with HrasD12 mutations at G2 construct (KwH2) were constructed through PCR using primers that would induce the necessary mutations in the Kras. The KwH1 construct used a primer designed by Craig Stropkay to induce the necessary point mutations at the G1 domain. A glutamine (Q) was inserted for a histidine (H), a glutamic acid (E) was changed to aspartic acid (D), and the proline (P) and serine (S) amino acids were changed to two amines (A); these were located roughly between a little after amino acid 90 and a little after amino acid 120. The KwH2 induced mutations at the G2 domain where four amino acids were changed. An aspartic acid (D) was converted to a glutamic acid (E), a threonine (T) was converted to a serine (S), a lysine (K) was converted to an arginine (R); these were continuous amino acids located a little bit before amino acid 135. The last mutation induced for KwH2 was located right after amino acid 140, where the phenylalanine (F) was converted into a tyrosine (Y).

Figure 2. MSCV-GFP-Ires-myc-NRasD12 vector representation with enzyme cut sites.
After the PCR the KwH1 and KwH2 fragments were inserted into a MSCV-GFP-ires vector, which looks very similar to the diagram shown above. The KwH1 and KwH2 fragment was inserted into the vector using a Not/Cla enzyme digestion and ligation.

Results
The difference in lethality between AML and CMML raised the question of why this happens. A key difference between the two is that AML blocks differentiation from bone marrow cells to peripheral blood cells and only keep proliferating GMP cells. CMML on the other hand does not undergo this differentiation block, and proliferates as specialized peripheral blood cells such as monocytes. The blocked differentiation is a major factor in why AML is so much more potent and lethal than CMML.

![Hematopoietic stem cell differentiation process]

*Figure 3. Hematopoietic stem cell differentiation process.*

Experiments found that Nras, Kras, and Hras all produced varying phenotypic and lethal effects. Hras produces AML, Kras produces CMML, while Nras produces both AML
and CMML. Therefore in order to look further into the genetic differences between AML and CMML, Hras and Kras were used to locate the genetic location causing the difference between the two different leukemias.

<p>| Table 1. Summary of hematopathologic characteristics of oncogenic NRAS, KRAS, and HRAS mice |
|---------------------------------|--------|----------|----------------|---------------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Construct</th>
<th>No. animals</th>
<th>Disease</th>
<th>Latency (d)</th>
<th>WBCs, 10^9/mL</th>
<th>Hematocrit (%)</th>
<th>Liver weight (g)</th>
<th>Spleen weight (g)</th>
<th>Pulmonary hemorrhage</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUG</td>
<td>3</td>
<td>None</td>
<td>N/A</td>
<td>6–12</td>
<td>53.6 ± 3</td>
<td>10 ± 0.1</td>
<td>0.1 ± 0.04</td>
<td>—</td>
</tr>
<tr>
<td>MUG/KRAS</td>
<td>4</td>
<td>AML</td>
<td>45.6</td>
<td>6–16</td>
<td>31.4 ± 5</td>
<td>17 ± 0.3</td>
<td>0.8 ± 0.2</td>
<td>—</td>
</tr>
<tr>
<td>MUG/KRAS</td>
<td>4</td>
<td>CMML</td>
<td>62.5</td>
<td>45–100</td>
<td>26.5 ± 4.7</td>
<td>19 ± 0.2</td>
<td>1.0 ± 0.45</td>
<td>—</td>
</tr>
<tr>
<td>KRAS</td>
<td>9</td>
<td>CMML</td>
<td>64</td>
<td>14–73</td>
<td>27 ± 6</td>
<td>17 ± 0.5</td>
<td>0.9 ± 0.1</td>
<td>—</td>
</tr>
<tr>
<td>KRAS/HRAS</td>
<td>9</td>
<td>CMML</td>
<td>40</td>
<td>14–57</td>
<td>20 ± 4</td>
<td>1.45 ± 0.3</td>
<td>0.9 ± 0.1</td>
<td>—</td>
</tr>
<tr>
<td>HRAS</td>
<td>9</td>
<td>AML</td>
<td>43</td>
<td>8–24</td>
<td>21 ± 10</td>
<td>1.4 ± 0.3</td>
<td>0.7 ± 0.2</td>
<td>—</td>
</tr>
</tbody>
</table>

*Median latency within the particular group.
*WBC count is given as the range of WBCs for the disease state within a particular disease group.
*Hematocrit, liver, and spleen weights are average ± SD.
*Mouse transduced with vector control; MCSV-NRAS-GFP (MUG).
*Not applicable.
*High dose.

*Figure 4. Summary of hematopathologic characteristics of oncogenic Nras, Kras, Hras in mice (Parikh, et al 2007)*

The first idea was to swap the hypervariable region located in the tail region of Hras and Kras considering this area had the most differences. By swapping the hypervariable regions of the two and performing a bone marrow transduction/transplantation, it could be tested to see phenotypically what kind of leukemia arises. If the mice developed AML then they were expressing Hras qualities, while if the mice developed CMML then that must mean they were expressing Kras. Therefore Hras with a Kras hypervariable region (HK) was constructed and a Kras with a Hras hypervariable region (KH) was made.
The results showed that HK very closely resembled Hras phenotype. This meant that the K tail did not have the genes needed to induce the necessary changes. KH on the other hand produced a hybrid Hras/Kras phenotypic expression. That means the H tail is most likely inducing some strange effects and would be inconclusive for our purposes. Also to confirm that the percentage of RBC at death showed that HK closely resembled Hras, which meant it was still exhibiting AML, while KH did not have the same effect since it
was definitely lower than the amount for Kras. Therefore our experiment takes the next step, by using Kras, since its tail did not have any effect on the phenotype expressed. So Kras is used to further isolate the location of the genes that give Kras and Hras its properties, since it isn’t on the Kras hypervariable region. That hypothesis became the backbone of this experiment, since by ruling out the hypervariable region as the cause for the phenotypic difference between Hras and Kras, it only left several more mutations to be tested. It was decided to split the remaining mutations in two groups, the G1 domain (first four different amino acids) and the G2 domain (last four different amino acids before the hypervariable region). Using the Kras as the backbone, Hras mutations were induced on the Kras at either the G1 or G2 domain. Therefore KwH1 represented Kras with Hras mutations induced at the G1 location, while KwH2 represented Kras with Hras mutations induced at the G2 location. This will allow us to narrow down the location of the gene or genes responsible for the difference between Hras and Kras. As shown in the data section, PCR was used to generate the mutations needed in our Ras constructs. After creating the new Kras hybrids it was combined with the MSCV-ires-GFP vector to be expressed in Balb/c mice. That is the point that we have reached and are currently waiting to infect recipient Balb/c mice with our new constructs and multiple control vectors.

**Discussion**

The next few weeks will be spent monitoring the health and effects of the mice that are infected with the bone marrow cells containing the virus that was produced by our constructs. The phenotypic expression of the type of leukemia along with other
factors such as WBC or RBC count will help reveal the location of the amino acids responsible for the blocking of cell differentiation. If the KwH1 mice exhibit symptoms of AML then we know that one of the four amino acids isolated in the G1 domain is the cause for the expression of oncogenic Hras and the prevention of cell differentiation. The same would go for KwH2. However if both of them exhibit AML properties it could mean that the Ras needs multiple amino acids in order to change its properties. If neither exhibits AML then there might be something more complicated taking place within the different Ras protein and genes. The success of this experiment with the expression of AML, would require another experiment to pinpoint exactly which gene is contributing to the change in Ras. The more that can be discovered about Ras the better chance that we can understand a major cause of leukemia. The better knowledge we have of Ras, especially information, which can allow us to isolate the problem that triggers a more lethal or mild version of the leukemia would be a great breakthrough. It can be of vital importance to help provide an alternative treatment by weakening the severity of the leukemia or possibly even find a more permanent treatment.

One thing that has to be kept in mind when tests are performed regarding Ras as an oncogene in mice, is that the gene expression and genomic makeup are so different between humans and mice. H-ras in humans are very rare, and do not always lead to leukemia, however in mice it is a very powerful oncogene in inducing myeloid leukemia. This does not mean that the research is not worthwhile, it just demonstrates how much is still left to be discovered about Ras. Especially since these experiments on mice are used as models of the disease but may not necessarily reflects what happens in humans. The pathways alone would take a long time to completely understand as will understanding
better about the structure and configuration of the different Ras forms. Knowledge and
technology increase exponentially, in the last decade or even years our knowledge about
the human body and genome has increased dramatically. Understanding Ras is only the
first step in the treatment for cancer, but it would be a vital step in the right direction.
Neil Armstrong summed it up the best with his famous quote, "That's one small step for
man, one giant leap for mankind".
References


