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作品名稱：氧化壓力影響基因轉換表現對脂肪分化之作用

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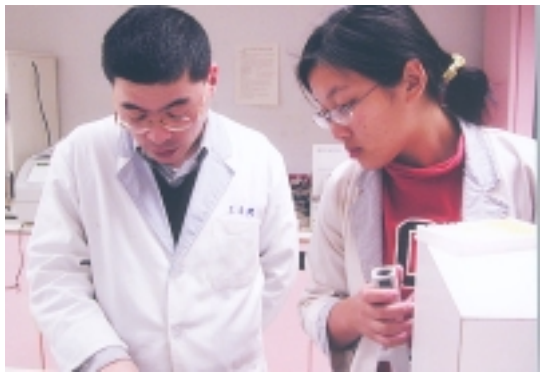

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我是楊媄涵，來自有草根性的南部，父親的老家住鹿草。父母都是老師，還有一個妹妹就讀國小。我在國小五年級曾利用一個月做科展得到佳作。很高興這一次能利用甄試上高中到進入高中的半年，參加醫學健康研究，覺得更有趣。我們解答一些脂肪分化的機轉問題，但也留下更多等待解答的問題。這是和書本上知識有些不同的地方! 課本上的知識大多已有結論或歸納過的知識。研究的結果總是和假說不完全相同；而且前因後果一下子分不清楚。這樣的研究經驗，會教人行事作爲的思想更周密；也會教人有學海無涯的心理準備；更叫人相信 21 世紀必定有許多有趣生物科技與健康科技等著我們去開發。

氧化壓力影響基因轉換表現對脂肪分化之作用

壹、中文摘要：

細胞脂肪分化是造成肥胖、骨質疏鬆、和糖尿病的重要前置因素。我們若要維持良好身材又想省去減肥藥的問題，那麼我們必須了解造成脂肪化的原因，才可能擁有好的預防之道。利用人類骨髓間質細胞可以分化成骨質與脂肪等細胞的特性，我們研究氧化壓力對間質細胞 Ras 基因轉換表現後骨質與脂肪分化的影響。結果發現以添加超氧根 (O_2^-, 15 nM)形成氧化壓力，可促進正常 Ras 基因表現的間質細胞朝骨質分化；相反地，超氧根會促進 Ras 基因突變而不表現的細胞，朝向脂肪分化的現象。進一步研究其作用原理，發現氧化壓力可促進 Ras 蛋白質啓動細胞外訊息活化酵素(ERK)，接著驅動骨質轉錄因子(CBFA1)表現，再到骨鈣蛋白質與骨結節形成。而抗氧化酵素(超氧根轉化酵素; SOD, 500 U/ml)的作用，可以抑制正常 Ras 基因細胞氧化壓力下骨質分化的進行；但不能防止氧化壓力促進 Ras 基因突變細胞，朝向脂肪分化的作用。總結而言：Ras 基因的表現與否，是決定脂肪分化的關鍵切換點；也是影響氧化壓力對間質細胞朝骨質分化的樞紐。這種基因與氧化壓力互動影響骨質與脂肪分化的剖析，將有助於提醒人們：使用抗氧化劑來調節抗衰老、肥胖、和美容時，必須是在不同情況和不同基因體質的人，有所不同。

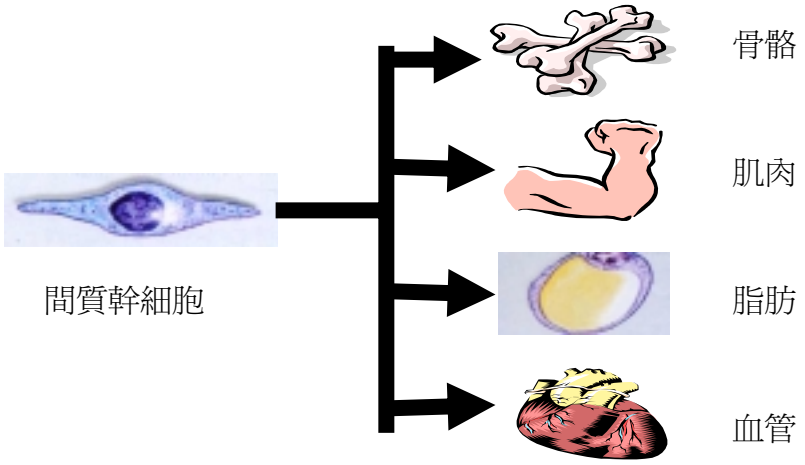
貳、研究背景與動機：

一. 脂肪化常是肥胖和慢性病的前奏

1. 現代人都怕肥胖和慢性病，有人甚至使用偏方減肥預防慢性病。
2. 從了解脂肪化機轉著手，人們或許可遠離減肥藥，遠離慢性病。

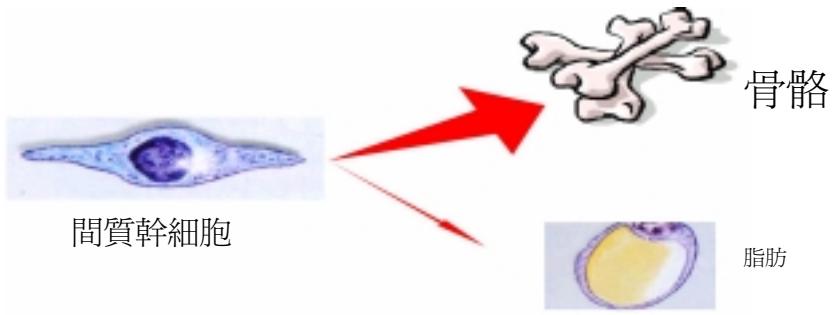
二. 脂肪細胞可切換成骨骼、肌肉、與血管等細胞

1. 目前已知脂肪細胞是由間質幹細胞演變而來的。
2. 間質幹細胞可分化爲脂肪、骨骼、肌肉、與血管等細胞(1-3)。



三. 學習掌握脂質分化，可以造就健康，遠離肥胖和減肥藥

1. 我們關心肥胖、骨質疏鬆、以及減肥藥傷害的問題。
2. 學習掌握間質細胞分化是人體細胞新陳更替不可或缺的泉源。也是避免肥胖、維持健康的方法(4,5)。



參、研究目的：

愈來愈多的證據顯示，不同基因的表現和不同生長素的消長與脂肪化最爲相關 (6)。而且氧化壓力與肥胖、骨質疏鬆、糖尿病和高血壓也有關係(7,8)，所以我就利用甄試上高中後的半年空檔時間，選擇使用氧化壓力與基因轉換表現影響間質細胞脂肪分化進行研究。實驗是使用不同 Ras 基因轉換表現 (Ras-dominant-negative expression)的骨髓間質細胞株，來研究環境中氧化還原的改變，對有無 Ras 基因表現的間質細胞脂肪分化的影響。期待透過調節氧化壓力或改造基因的表現來控制間質細胞脂肪分化，並達成下列三項特定目標:

一. 探討人類骨髓間質細胞在體外培養與分化的狀況，包括細胞生長以及細胞形態分化改變的觀察。

二. 接著以轉換 Ras 基因表現的模式，研究間質細胞在特定基因表現異常時對間質細胞脂肪分化的影響。

三. 研究外加氧化壓力與相對抗氧化劑，對 Ras 基因轉換表現的間質細胞脂肪分化的影響：包括細胞內信息傳遞與細胞形態分化改變的機轉。

透過這些研究結果，將可以幫助我們了解不同基因表現的細胞在氧化壓力環境下，如何影響細胞內信息傳遞和調控細胞分化。利用這些調控原理，我們或許可以對不同基因體質的人，藉由外加氧化還原劑來避免間質細胞脂肪化的進行，達到促進健康的目的。

肆、研究材料與方法：

一、*間質細胞的培養：* 選用人類骨髓間質細胞株(HS-5; $1x10^5$ cells/ml)，於含 10%胎牛血清的(Dulbecco modified Eagle medium; DMEM)培養基中，每三天換一次培養基來進行。

二、*間質細胞分化鑑定：*間質細胞培養於不同情況 15 天後進行分化鑑定(9):

1. 辨別骨質細胞分化-是以銀染測定骨結節(bone nodule)形成爲準。
2. 辨別肌肉細胞分化-是以抗體偵測肌球蛋白(myotubulin)表現爲準。
3. 辨別軟骨細胞分化-是以軟骨醣蛋白(chondrotin proteoglycans) 偵測。
4. 辨別脂肪細胞分化-是以油紅(Oil Red O) 的染色偵測爲準。

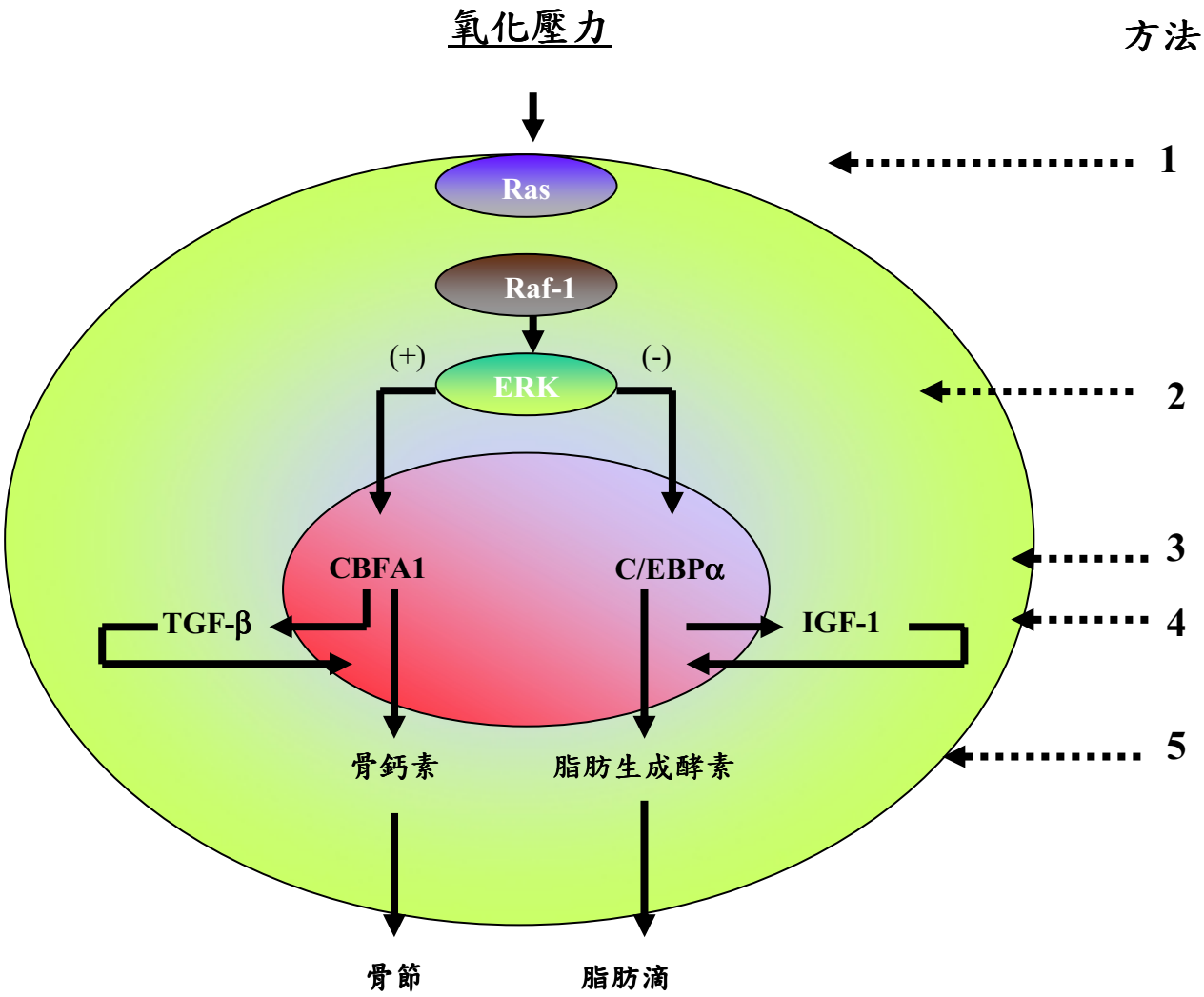
三、*轉換 Ras 基因的正負表現(Ras-dominant negative expression)：*正常原生細胞株帶有正常的 Ras 基因表現，另外以油脂體(liposome; FuGENE™; Roche Diagnostic Corporation, Indianapolis, IN, USA) 把一種帶有突變 Ras 基因(Asn-17 *ras* ^{H})植入間質細胞後，使細胞強勢表現突變 Ras 基因而異常表現正常 Ras 基因(dominant negative expression)。這樣的基因轉變所造成的間質細胞分化上的改變，如果不同於 Ras 正常表現細胞的結果，則可推論 Ras 基因表現與脂肪化的作用。

四、*氧化與抗氧化調控間質細胞的分化：*

1. 氧化壓力是以 Xanthine 氧化酵素(250 μU/ml)作用於 Hypoxanthine (10 mM)產生的超氧根(O_2^-; 15 nM)進行研究。
2. 抗氧化作用則是加入超氧根轉化酵素(superoxide dismutase, SOD: 500 U/ml)於細胞培養液中進行。

這些氧化壓力與抗氧化作用於細胞的結果，會先改變其細胞內訊息傳遞，然後改變細胞外生長素分泌，以及細胞脂肪化形態的出現。

五、*間質細胞分化訊息傳遞研究設計：*研究是以外加氧化還原劑作用於有無 Ras 基因表現細胞，如何影響其細胞內訊息傳遞進行如下圖:



註：方法１，２，３，４和５分別描述於下一頁

六、*細胞內訊息傳遞的測定方法:* 訊息蛋白質的測定是將培養於不同情況的細胞($2x10^6$/ml)，以磷酸鹽緩衝液(PBS)清洗後，在 4℃低張的細胞分解液(10mM Tris buffer, 含 2 mM $MgCl_2$, 10mM KCl, 0.1 mM EDTA 和 0.7% NP-40)分解細胞 30 分鐘。然後細胞質與細胞核，可在低度離心(500 g)5 分鐘後，被區分出來。下層的細胞核再次經含 1% NP-40, 1mM PMSF, 2 μM DTT, 2 μg/ml leupeptin 的 Tris 緩衝分解液作用 30 分鐘。這些分解後細胞核與細胞質中的蛋白質可以用高度離心的方法(12,000 g)10 分鐘沉澱取得，並用標準測定蛋白質濃度測定組(Bio-Rad assay kit)定量濃度後進行以下研究：

1. 測定 Ras 蛋白質活化，是用具親和力的受質 Raf-1 偶合瓊脂去沉澱活化的 Ras 蛋白質，再用抗 Ras 抗體進行西方墨點電泳分析(9)。研究是取 500 μg 胞質蛋白質與 Raf-1 偶合瓊脂反應、沉澱，再以 12% 蛋白質電泳(polyacrylamide gel electrophoresis; PAGE)，之後轉移蛋白質到 nitrocellulose membrane 上以 1:2000 抗 Ras 抗體進行西方墨點分析。

2. 測定 ERK 的活性以其受質 MBP(myelin basic protein)被磷酸化的特性，再以抗體測定磷酸化受質含量(10)。即是取 500μg 胞質蛋白質與抗 ERK 抗體(2.5 μg)反應，再以會抓免疫複合體的 Protein A 沉澱出 ERK 蛋白(immunoprecipitate)。之後把沉澱到的 ERK 蛋白質與 MBP(myelin basic protein)進行磷酸化反應(Mg^{++}-ATP, MBP; 購自 Upstate Biotechnology Inc.)30 分鐘。這些反應物再以 12% SDS-PAGE 跑電泳，並轉移蛋白質到 nitrocellulose membrane 上以 1:2000 抗磷酸化抗體進行西方墨點分析磷酸化 MBP 代表 ERK 活性。

3. 骨質轉錄因子(CBFA1)和脂肪轉錄因子(C/EBP α)測定，則是取 500 μg 細胞核蛋白質與其特定抗體(2.5 μg)進行免疫沉澱取得其特定核內轉錄因子 (10)，再用 12%SDS-PAGE 跑電泳，之後轉移蛋白質到 nitrocellulose membrane 上以 1:2000 特定抗體進行西方墨點分析 CBFA1 和 C/EBP α 含量。

4. 生長素 TGFβ 和 IGF-1 含量是以免疫酵素法(ELISA)測定。以取自細胞培養液 100ul，用購自 R&D 公司的免疫酵素法(ELISA)測定組測定(9)。

5. 骨質和脂肪細胞分化成熟指標骨鈣蛋白質與脂肪生成酵素的測定則是用定量粹取自細胞質蛋白質(30 μg)來跑電泳後，把電泳膠的蛋白質轉印到 nitrocellulose membrane，再用帶有化學光受質的特殊抗體去尋找其表現含量。

伍、研究結果：

一. 人類骨髓間質細胞體外培養與生長：

研究首先以細胞計數格計算培養後的人類間質細胞株細胞，將其細胞數調整爲每毫升 1×10^5 細胞（圖一、左上圖），再將細胞培養於含有 10%胎牛血清的基本培養基中，置入培養皿中（圖一、右上圖），於 37℃，5%二氧化碳培養箱培養。這些細胞在第三天更換新培養基時，可看到其沾黏生長在培養皿中呈類纖維細胞（圖一、中圖）。這些細胞經過每三天更換一半培養基，到了 15 天，用胰蛋白酵素（trypsin, 0.1%）把細胞收集下來，進一步鑑定其分化形態。

二. 人類骨髓間質細胞分化爲骨質、軟骨和肌肉細胞的形態鑑定：

將體外培養 15 天的間質細胞($1x10^5$/ml；0.1ml)，用細胞離心模片方式散播於打到玻片上。接著用甲醇固定後，以不同形態染色方法鑑定細胞分化，並計算其屬性分佈狀況。在重覆三次的實驗中，這些間質細胞可分化成銀染 (von Kossa staining)陽性的骨質細胞(圖一、下圖左一)，平均比例爲 48.2%; 分化成軟骨醣基質陽性的軟骨細胞(圖一、下圖左二)，比例是 31.6%；分化成肌球蛋白陽性的肌肉細胞(圖一、下圖右二)，比例爲 21.2%；但看不到油紅染色陽性的脂肪細胞(圖一、下圖右一)。

三. 骨髓間質細胞 Ras 基因表現轉換的操作與其作用結果：

由於細胞膜上的 Ras 蛋白質是細胞分化的重要分子，也是調節間質細胞脂肪分化的因子(11)。因此我們利用強勢負向轉換(Ras dominan-negative transformation)的方法，讓細胞的 Ras 基因突變後而不表現 Ras 蛋白質來研究。這種沒有 Ras 基因表現的間質細胞分化，正好可以拿來對照正常 Ras 基因表現時的分化結果；從而了解 Ras 基因表現與否在環境因素改變時，其細胞分化行爲如何改變。操作 Ras 基因轉換的原理如圖二所示：先是把有突變的 Ras 基因(Asn-17 ras^H)，用基因攜帶媒介(FuGENE™; Roche Diagnostic Corporation, Indianapolis, IN, USA) 植入間質細胞，去替代正常 Ras 基因。這些細胞的 Ras 基因是否轉換成突變的 Ras 基因，是藉由突變的 Ras 基因同時帶有 G418 抗藥基因來篩選。當我們在培養液中加入細胞毒殺藥劑(G418)時，含正常 Ras 基因的細胞會被殺死；然而 Ras 基因突變的細胞因同時帶有抗藥基因，所以會被選擇持續生長。檢測基因轉換的結果，發現原有間質細胞會正常表現 Ras 蛋白質；但突變基因轉換過的細胞則沒有活化 Ras 蛋白質的表現。

四. 氧化壓力下 Ras 基因表現轉換後的間質細胞分化型態不相同：

當外加由超氧根(O_2^-)產生的氧化壓力時，對正常 Ras 基因表現的間質細胞有促進骨質細胞生長分化的作用，如圖三、左上圖所示，細胞培養 15 天後在肉眼下觀察，有 80%細胞分化成有骨結節形成的形態；但對脂肪細胞分化沒有促進作

用。在顯微鏡下觀察，氧化壓力確實可以增加骨結節形成的染色；但沒有油紅染色陽性的脂肪細胞出現(圖三、左下圖)。當 Ras 基因突變而不表現時，細胞本身就有傾向脂肪分化的現象。在外加超氧根形成的氧化壓力時，不但沒有骨質細胞的形成，反而加速增多脂肪分化的現象(圖三、右上圖)。在顯微鏡下觀察也是類似：氧化壓力不但沒有增加骨結節形成的染色；反而增加油紅染色陽性的脂肪細胞出現(圖三、右下圖)。

五. 氧化壓力作用下不同 Ras 基因表現的間質細胞分泌不相同生長素：

除了觀察細胞分化型態的變化，我們也分析在不同形態分化下，間質細胞分泌生長素的情形。我們選擇測定與骨骼和脂肪分化有關的生長素 TGF β 和 IGF-I 的分泌研究。結果如表一所示：氧化壓力下，可促進正常 Ras 基因表現的間質細胞分泌骨質生長較相關的 TGF β 生長素。相反地，氧化壓力作用，則促進 Ras 基因突變後不表現的間質細胞，分泌與脂肪分化較相關 IGF-I 生長素。

表一　超氧根影響 Ras 基因正常與突變細胞骨質及脂質生長素分泌

細胞生長素（pg/ml）	Ras 正常表現細胞			Ras 突變細胞		
	超氧根 (-)	超氧根* (+)	超氧根(+) 超氧轉化酵素	超氧根 (-)	超氧根* (+)	超氧根(+) 超氧轉化酵素
TGF-β; 平均值	145.5	426.8**	168.5**	182.6	185.4	175.4
(標準誤)	(23.4)	(39.3)	(27.4)	(18.4)	(27.4)	(22.6)
IGF-1; 平均值	86.3	93.8	95.8	178.2	286.4	292.4
(標準誤)	(14.6)	(17.4)	(20.2)	(19.6)	(26.2)	(19.6)

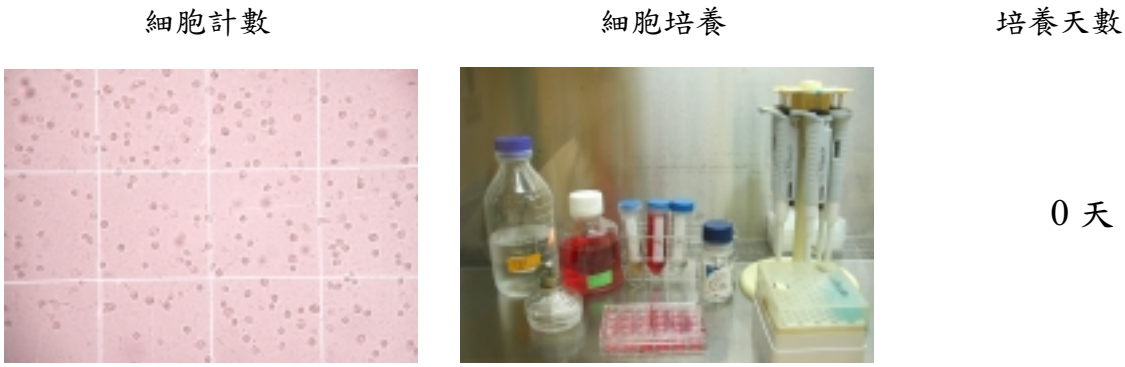
註解： Ras 正常與 Ras 突變細胞在不同超氧根與超氧轉化酵素作用下培養三天後，收集培養液以酵素免疫抗體測定組(ELISA)測定骨質生長素(TGF-β)及脂質生長素(IGF-1)。
* 超氧根(O_2^-)是以 10 mM hypoxanthine + 250 μU/ml xanthine oxidase 生成 15 nM 進行。
**超氧轉化酵素(SOD; 500 U/ml)有意義的($p<0.01$, t test)抑制超氧根促進 TGF-β之產生。

爲了進一步證實這個不同作用確實是超氧根氧化壓力作用的結果，我們用超氧根轉化酵素(SOD; 500 U/ml)去中和氧化壓力作用。結果確實可以看到正常 Ras 基因表現的間質細胞分泌 TGF β 生長素可以被抑制下來；然而 Ras 基因突變的間質細胞，其氧化壓力促進 IGF-I 生長素的分泌則沒有被抑制下來。顯然表示在不同 Ras 基因表現下，氧化壓力誘導不同的生長素分泌也隨之改變。

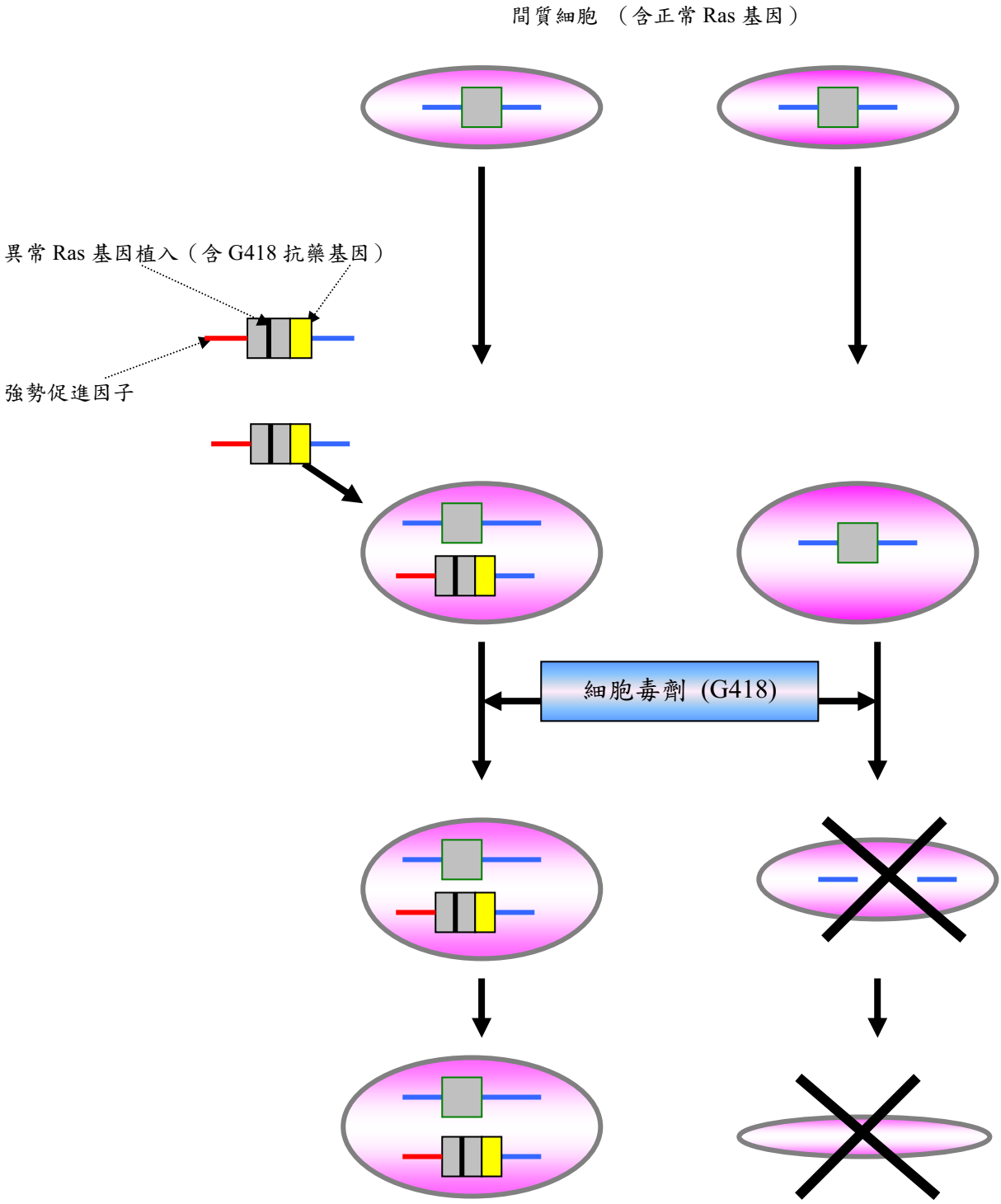
六. 氧化壓力和抗氧化劑對不同間質細胞分化的作用機轉：

進一步研究不同 Ras 基因表現的間質細胞在氧化壓力與抗氧化酵素作用下的訊息變化。結果發現正常 Ras 基因表現的間質細胞在氧化壓力作用下會激發細胞外訊息活化酵素(ERK)的活化，接著骨質特殊轉錄因子(CBFA1)的表現增加，再到骨鈣蛋白質的表現，以及形態上可辨識的骨結節形成表現(圖四，左圖)。這些氧化壓力作用，可以因爲加入特殊抗氧化酵素(SOD)而受到抑制(圖四，左上三列)。相反地! 當 Ras 基因突變而沒有表現時，外加氧化壓力不會誘導 ERK 和 CBFA1 的表現，也不會有骨鈣蛋白質和骨結節形成；卻增加脂肪特殊轉錄因子(C/EBPα)與脂肪生成酵素的表現(圖四，右上圖)，並且分化爲油紅染色陽性的脂肪油滴表現細胞。有趣的是，這種增加脂肪化的訊息，不會在加入抗氧化劑之後被中和掉(圖四，右下圖)。顯然氧化壓力對正常 Ras 基因表現的細胞訊息傳遞是確定由 Ras-ERK-CBFA1-骨鈣蛋白質到骨結節形成；而抗氧化酵素(SOD)的作用，可以抑制骨質分化進行。但是氧化壓力對 Ras 基因突變而不表現的細胞分化的影響，則是一種不可逆的作用，抗氧化酵素(SOD)的使用，不能防止其脂肪分化進行。

圖一 人類骨髓間質細胞培養與形態鑑定

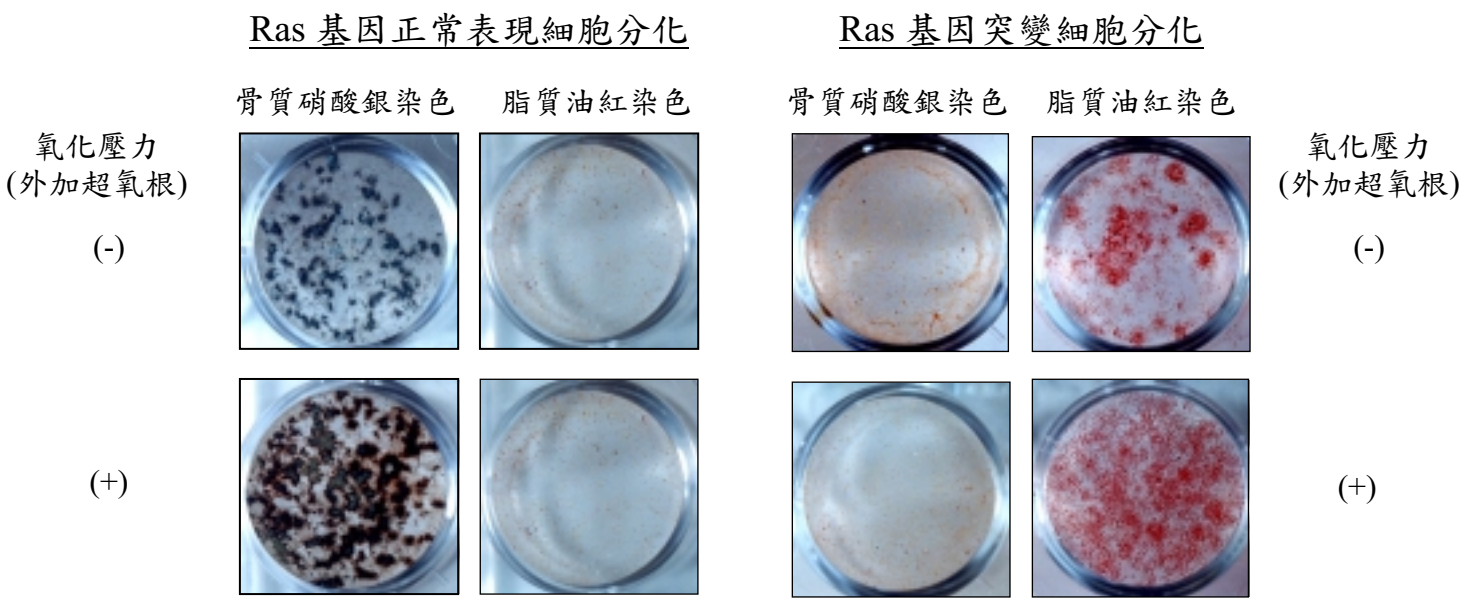


圖二 骨髓間質細胞 Ras 基因突變轉換表現的操作原理

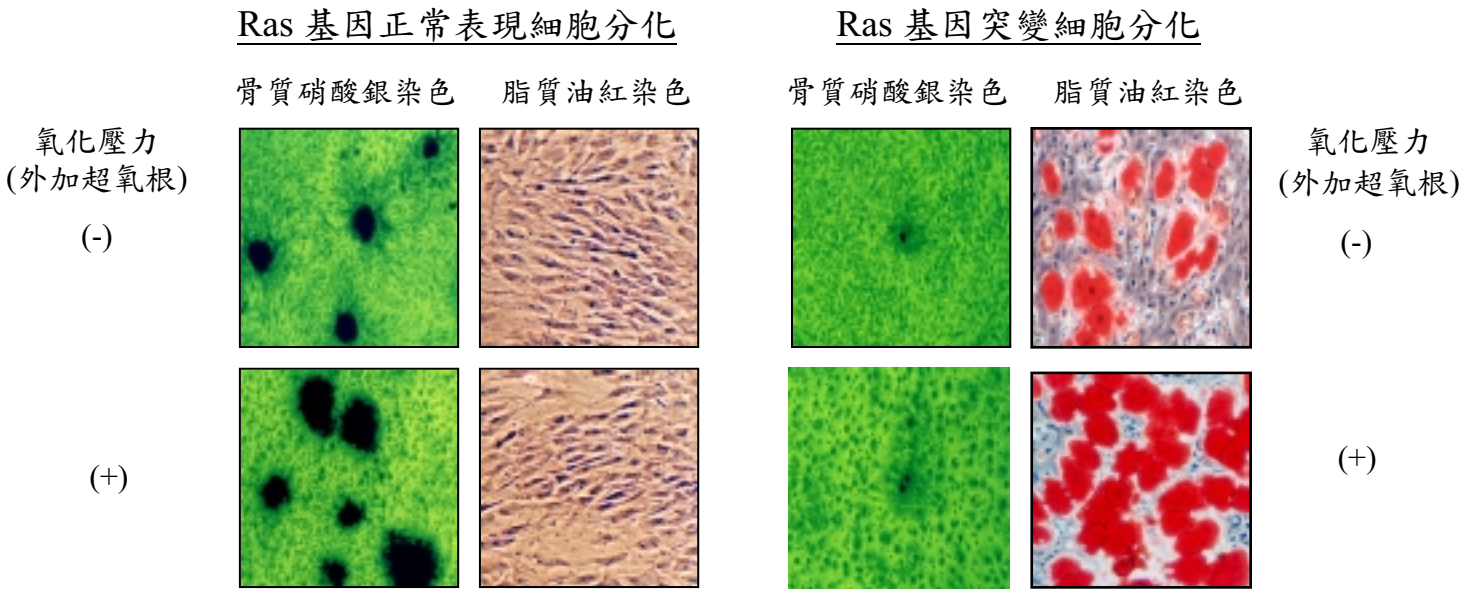


圖三 氧化壓力影響 Ras 基因轉換表現的骨髓間質細胞分化

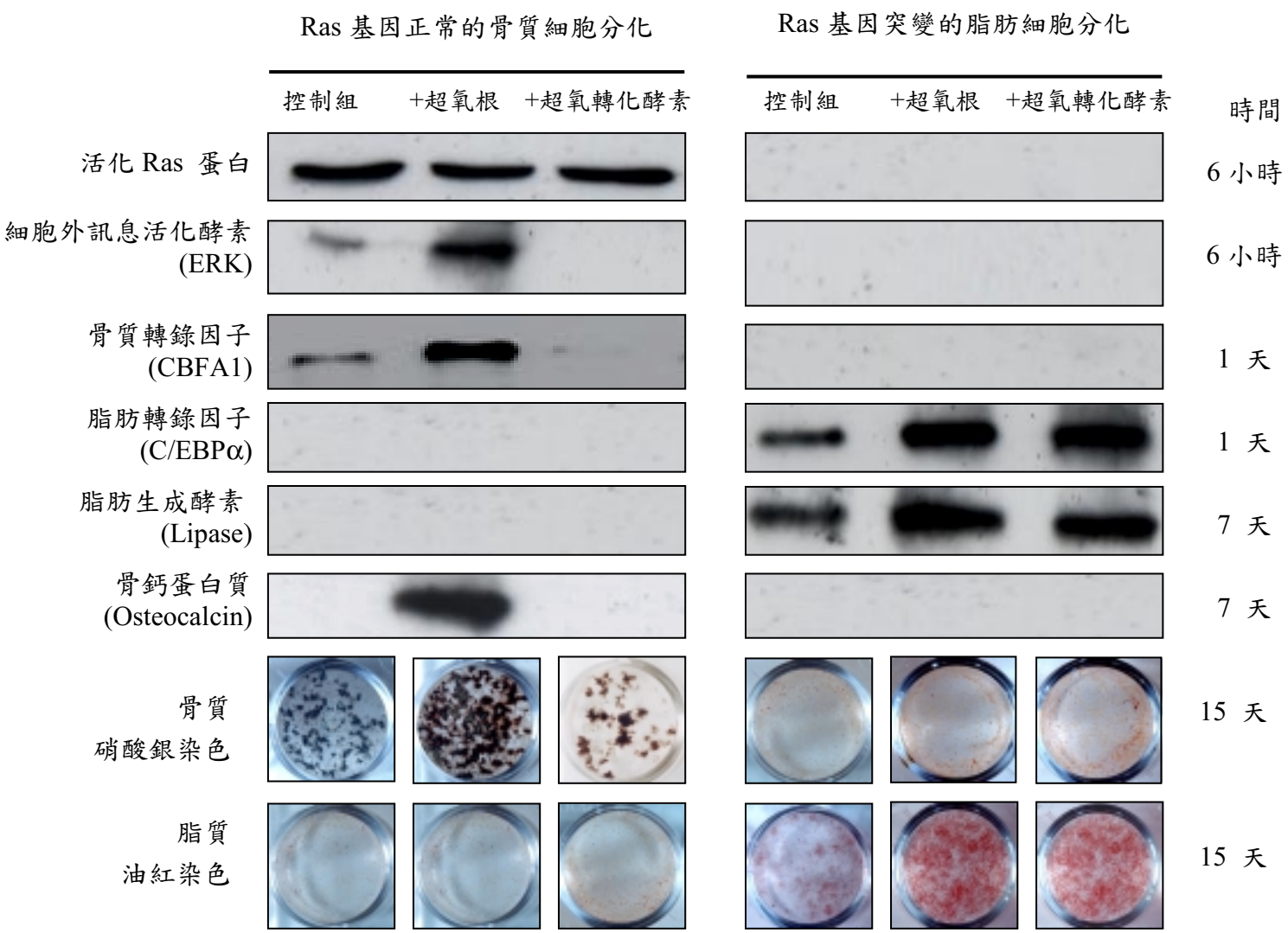
A. 肉眼(Gross)下的間質細胞分化



B. 微觀(Microscopic)下的間質細胞分化



圖四 超氧根影響 Ras 基因突變細胞骨質與脂質分化訊息變化



陸、研究討論：

一. 重要發現:

在體外培養人類間質幹細胞的模式，我們發現 Ras 基因的表現與否是決定間質細胞轉換骨質與脂肪分化的分水嶺。氧化壓力的作用在正常狀態下對人類間質細胞而言，是促進骨質分化的推動力；然而在 Ras 基因突變後負向不表現時，氧化壓力不會促進骨質生成，反而推動脂肪分化作用的進行。這個有趣的發現顯示：使用抗氧化劑來預防慢性疾病，在不同疾病和不同基因體質的人上，其結果不一定相同。

二. 釐清骨質與脂質分化不同訊息傳遞:

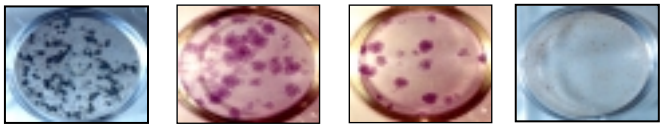
研究中也發現氧化壓力促成間質細胞分化爲骨母細胞的訊息傳遞順序是透過促進 Ras 蛋白質的活化，啓動 ERK 的活化，促進骨質特殊轉錄因子(CBFA1)表現與增加間質細胞生長素 TGFβ和成熟骨質素的呈現，再到最後骨結節的形成。這種機制的剖析，將有助人們選擇在不同訊息點的切入，來加強骨質的生成與分化，以便減少骨質疏鬆疾病。另外在操控 Ras 基因突變後而不表現時，ERK 的活化不被啓動的結果，則轉向促脂肪轉錄因子(C/EBPα)表現，造成脂肪分化，以及生長素 IGF-1 的分泌與脂肪酵素產生。這些訊息傳遞的釐清，或許有助促進骨質新生但減少脂肪分化的控制。利用這些原理，有可能比目前有人以骨形態蛋白質(bone morphogenetic protein)促進骨質新生的方法(12)，來得好。

三. 異於常理的發現適當氧化壓力有助骨質分化:

另外值得討論的是氧化壓力的存在不全然都是壞東西。傳統上認爲：氧化壓力作用多半誘發不好的細胞內訊息傳遞與結果，例如已知人類老化(Ageing)與氧化壓力有關(7,8)。然而我們在這個實驗卻發現，外加氧化壓力，有助正常間質細胞分化成骨質細胞的現象，這與老化的人容易骨質疏鬆的事實，並不符合。不符合的原因可能是因爲：**1**.適量氧化壓力有助於骨質增加，而過量或長期氧化壓力的累積作用，反而促成骨質疏鬆的結果。**2**.老化的細胞可能有些基因表現異常，致使在氧化壓力下的間質細胞會朝脂肪化進行。**3**.老化的間質細胞可能分泌較少的骨質生長素或較多的脂肪細胞生長素，使得脂肪分化增加。這些可能性都值得我們進一步釐清，才能有效提出老化後脂肪化與骨質疏鬆的防治之道。

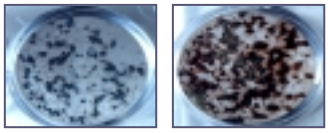
柒、結 論 (Conclusions):

一. 體外培養的人類間質細胞可分化成骨質、軟骨與肌肉細胞，但沒有脂肪細胞



骨母細胞　軟骨細胞　肌肉細胞　脂肪細胞

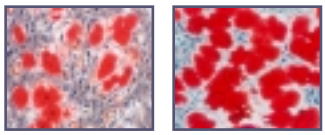
二. 外加氧化壓力促成正常 ras 基因的間質細胞朝骨質分化



(-)　(+)

氧化壓力 (15 nM)

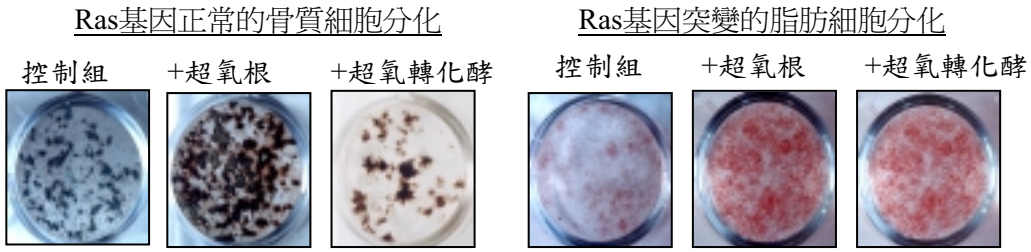
三. 當間質細胞因 Ras 基因突變不表現時，氧化壓力促進細胞朝脂肪方向分化



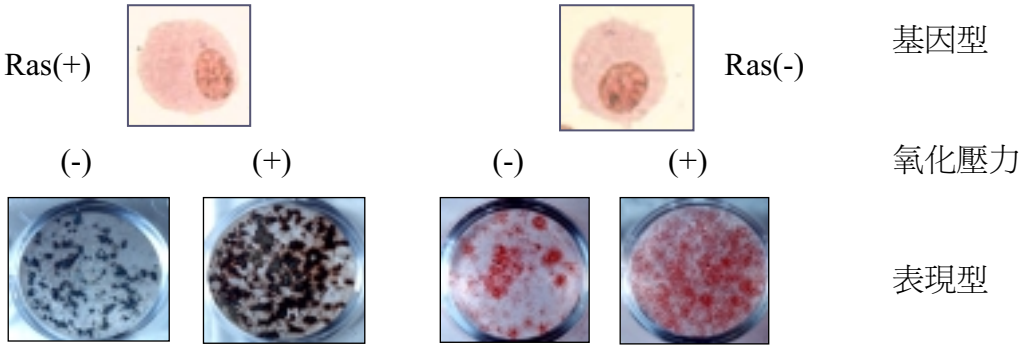
(-)　(+)

氧化壓力 (15 nM)

四. 使用抗氧化劑(SOD; 500 u/ml)可抑制外加氧化壓力促成正常 Ras 表現細胞朝骨質分化，但不會抑制外加氧化壓力促進 Ras 突變的間質細胞朝脂肪分化



五. 氧化壓力對不同 Ras 基因表現的間質細胞分化成骨質或脂肪的作用不一樣



捌、進 一 步 的 應 用 與 研 究:

一. 透過環境與基因的調控，人們有可能自己控制要長骨質或長脂肪!

二. 利用物理或化學的方法調控氧化還原反應，可以幫助把脂肪分化轉朝向骨質和肌肉分化。

三. 除了骨髓間質幹細胞可分化成骨質、軟骨、肌肉或脂肪，其他如臍帶血幹細胞和胚胎幹細胞都可分化成骨質、軟骨、肌肉或脂肪。所以我們也可以從不同角度切入去調控間質細胞的分化。

四. 我們正嘗試以氧化還原方法去影響臍帶血間質細胞分化的研究。初步結果很有趣！很歡迎大家一齊來研究或提供意見。我的 e-mail 地址是 ny_foxy@giga.net.tw

玖、研究致謝：

感謝指導教授王博士和實驗室裡的琦晶以及謙真大姊們，有他們指導培養細胞，減少污染才能漸漸有興趣。也謝謝研究區的所有大哥大姊們的幫忙指導準備培養基和緩衝液。更謝謝國中老師讓我有機會把握畢業前的日子參與研究；以及進入高中後，生物老師啓發式的教導。當然! 爸爸、媽媽和妹妹雅涵，你們給我的支持、鼓勵和督促也不容忽視。真的非常謝謝各位。我現在準備要朝向轉殖氧化還原基因去操控臍帶血間質幹細胞生長與分化研究去努力。初步結果很有趣，歡迎加入。 再次感謝大家，我愛你們！

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DIFFERENTIATION OF MESENCHYMAL STEM CELLS INTO BONE AND FAT CELLS, *IN VITRO*

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ABSTRACT

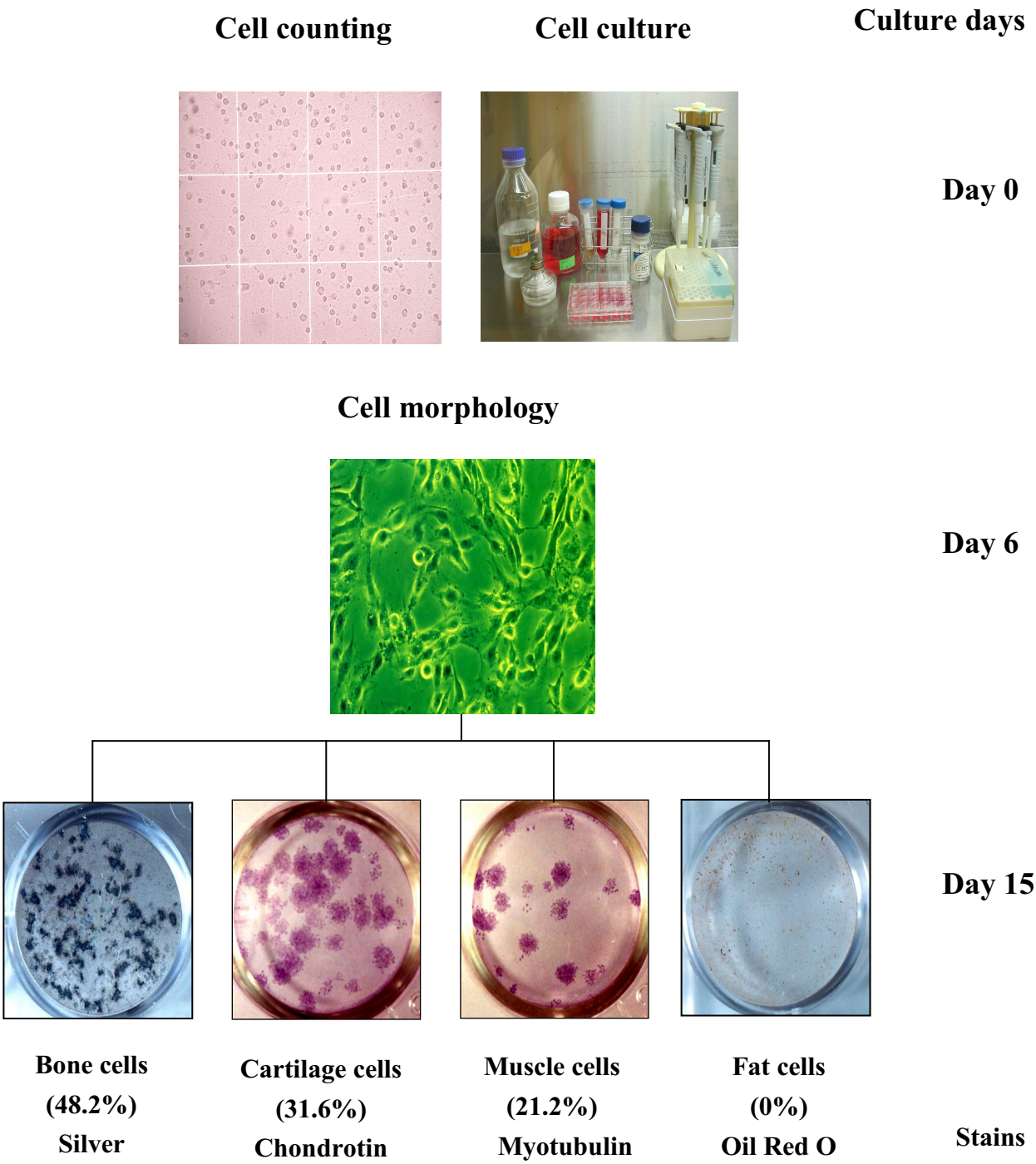
Human mesenchymal stem cells are able to differentiate into bone, muscle, cartilage or fat tissues. Our preliminary study with human mesenchymal cell line (HS-5) showed that HS-5 cells could differentiate to bone, cartilage and muscle but not fat cells as determined by histochemical staining of phenotypes. We have further studied the influence of oxidative stress on the switch between bone and fat cell differentiation.

Results showed that oxidative stress started with exogenous superoxide, produced by the interaction of xanthine oxidase and hypoxanthine, promoted the differentiation of osteogenic lineage showing expression of osteocalcin and bone nodule formations. The mechanism was investigated and superoxide was found to induce ERK (extracellular regulated signal kinase) activation; and then the expression of osteogenic specific transcriptional factor (CBFA1). A plasmid containing ras-mutant (Ser 17 Asn) which can inactivate the expression of ERK was transfected into the HS-5 cells for studying the influence of oxidative stress on ras-mutated mesenchymal cells. Surprisingly, it was found that oxidative stress did not promote osteogenesis but it enhanced adipogenesis from the ras-mutated HS-5 cells. Further studies indicated that superoxide neither induced ERK activation nor CBFA1 expression, but it did enhance expression of adipogenic specific transcriptional factor (C/EBPα) and lipoprotein lipase in the ras-mutated mesenchymal cells.

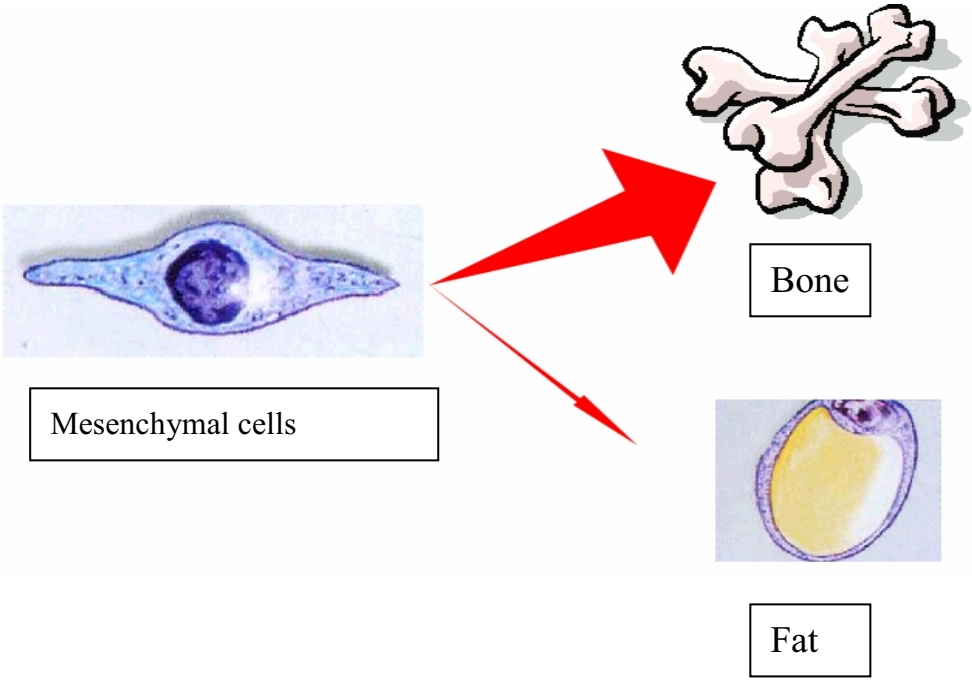
Taken together, the study model to induce the bone cell differentiation from human mesenchymal stem cells may be employed to make bone cells for tissue engineering.

Introduction:

Mesenchymal stem cells (MSCs) can grow into bone, cartilage, muscle, fat and other tissues (1). In a pilot study with human mesenchymal cell (HS-5) line, we found that HS-5 cells could differentiate to bone, cartilage and muscle but not fat cells as shown below:



Since Ras expression and superoxide have recently been implicated in mesenchymal cell differntiation (2,3), and switching of adipogenesis to osteogenesis in bone marrow has been proposed to treat osteopenic disorders (4). Employing an *in vitro* mesenchymal cell differentiation model, we have further studied how oxidative stress and ras gene expression modulate the bone cell differentiation in order to make bone cells for tissue engineering.



Results from this study model may provide better information applicable for bone tissue engineering by manipulating different gene expression and redox status in the future.

MATERIALS & METHODS

Materials:

Human bone marrow mesenchymal cell (HS-5) line was obtained from American Type Culture Collection (ATCC, Manassas, VA). A ras dominant negative mutant construct (Ser 17 Asn) was purchased from Upstate Biotechnology (Lake Placid, NY). The liposome transfection reagent, FuGene^{TM} 6, was obtained from Roche Diagnostics Co. (Indianapolis, IN). Aminoglycoside, G418, was acquired from Life Technologies (Gaithersburg, MD). Myelin basic protein, AgNO3, Oil Red O and Dulbecco's modified Eagle medium (DMEM) were from Sigma Inc. (Saint Louis, MO). Anti-Ras (Upstate Biotechnology), anti-osteocalcin (Biogenesis Technology, England, UK), anti-lipoprotein lipase (PROGEN Biotechnik, Heidelberg, Germany), anti-C/EBPα and anti-CBFA1 (Santa Cruz Biotechnology, Inc. CA) were used for Western blot analysis of signal transductions.

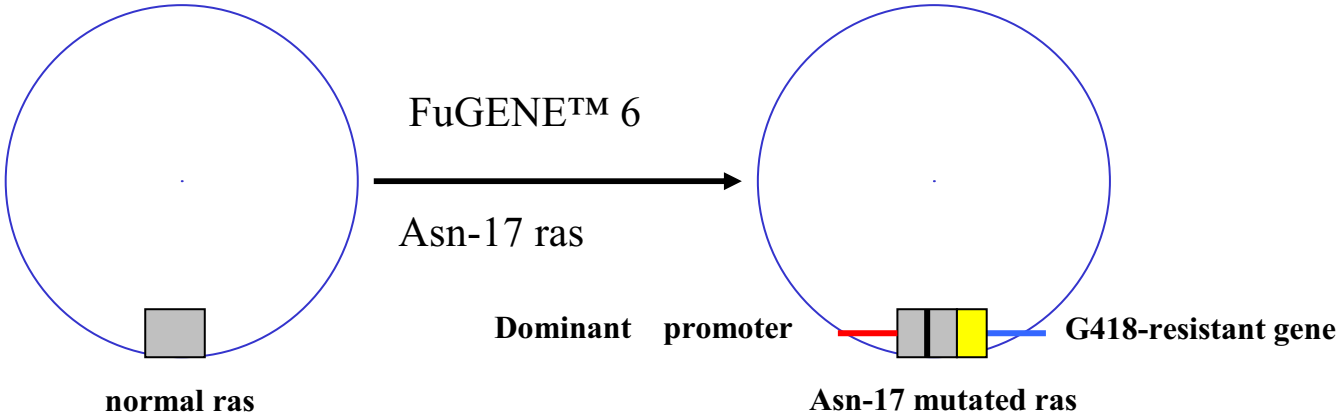
In vitro culture and differentiation of mesenchymal cells:

HS-5 cells ($5x10^5$ cells/well, 6 well-plate) were cultured in DMEM containing 10% fetal bovine serum at 5% CO_2, 37℃ incubator. One half of culture medium was replaced by fresh medium every 3 days. Cells were harvested by trypsinization and re-suspended in medium for studies. Silver staining (von Kossa staining), Oil Red O staining, and immunostaining of chondrotin proteoglycans and myotubulin were, respectively, utilized to identify bone, fat, chondrocyte and muscle cell differentiation (5).

Transfection of a dominant negative ras construct

Bone marrow HS-5 cells ($5x10^5$ cells/well) were transfected with 3 μg dominant negative Ras mutant cDNA plasmid (Asn-17 ras^H) using FuGENE™ 6 transfection

reagent according to the direction of the commercial kit. After removal of medium, the transformed cells were selected in the culture containing 400 μg/ml G418 (5).



Oxidative modulation of mesenchymal cell differentiation

Oxidative stress was induced by the superoxide production from the reaction of xanthine oxidase (250 μU/ml) with hypoxanthine (10 mM). The HS-5 cells incubated with this oxidative stress were cultured for 6 hours, 24 hours and 7 days for Western blot analysis of signal transductions (3,5). The culture supernatants from the cells with and without oxidative stress for 2 hours were harvested on day 3 for enzyme-linked immunoassay (ELISA) of growth factor (TGFβ and IGF-I) production. The cells with and without this oxidative stress were also subject to histochemical staining of cell differentiation after culture for 15 days.

Detection of signal transduction pathway by Western blot analysis :

Ras activation, ERK (extracellular signal regulated kinase) activation, osteogenic/ adipogenic transcription factor (CBFA1/ C/EBPα) expression, and cell differentiation markers (osteocalcin / lipoprotein (LP) lipase) were determined by Western blot analysis. Cells (1 x 10^7 cells) harvested from differently conditioned

cultures as indicated were lysed with 200 μl of the ice-cold buffer containing 10 mM Tris, pH 7.9, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.7% NP-40 on ice for 10 min. The cytosol was separated from nuclei by centrifugation at 500 μg for 5 min. The nuclei pellets were further lysed with a buffer containing 40 mM Tris, pH 7.9, 350 mM NaCl, 2mM $MgCl_2$, 1 mM EDTA, 0.2 mM EGTA, 20% glycerol, 1% NP-40, 1 mM phenylmethylsulfonyl floride, 2 μM DTT, 2μg/ml leupeptin and 1μg/ml aprotinin (Sigma Chemicals Inc., St. Louis, MN, USA) on ice for 20 min and harvested by centrifugation at 12,000 g, 4℃ for 10 min. Protein concentrations in the cytosolic and nuclear extracts were determined by Bio-Rad assay kit (Bio-Rad Laboratories, USA) (3,5). Protein samples from different conditions were loaded for determination of the signal cascade as described below:

A. Measurement of Ras activation was determined by Raf-1 affinity precipitation, followed by anti-Ras Western blot analysis of peroxidase-mediated chemiluminescence imaging. In brief, the activated Ras in 500 μg cytosol extracts were precipitated by Raf-1 agarose before loaded into 12 % SDS-PAGE. The activated Ras protein was recognized by the Ras antibody conjugated with horseradish peroxidase that could react with a substrate and develop a chemiluminescence for photo imaging (3).

B. Measurement of ERK activation, CBFA1 and C/EBPα expression was determined by immunoprecipitation with specific antibody, followed by Western blot analysis. In brief, crude cytosol or nuclei extracts (500 μg) were immunoprecipitated by specific antibody (2.5 μg) and pelleted with protein A agarose. The protein in the pellets was loaded to a 12 % SDS-PAGE and transferred to a nitrocellulose membrane for the Western blot analysis of peroxidase-conjugated chemiluminescence imaging.

C. Detection of bone and fat cell maturation markers (osteocalcin / lipoprotein (LP) lipase) were determined by Western blot analysis of osteocalcin and LP lipase

using specific antibodies. In brief, crude cytosol extracts (20 μg) were loaded to a 12 % SDS-PAGE and transferred to a nitrocellulose membrane for the Western blot analysis of peroxidase-conjugated chemiluminescence imaging.

Measurement of TGFβ and IGF-I production by ELISA

Culture supernatants from the HS-5 cells with and without oxidative stress for 3 days were harvested for measurement of TGFβ and IGF-I production as previously described (3).

RESULTS:

Transformation of ras-normal HS-5 cells to ras17 Asn-mutated HS-5 cells.

Human bone marrow mesenchymal stem cells (HS-5, $5x10^5$ cells/well) cultured in DMEM for overnight were transfected with 3 μg of the ras dominant negative plasmid containing a replacement of serine with asparagines at position 17. Since the Ser-17-Asn ras mutant also contains an antibiotic (G418) resistant gene, the transformed cells could be selected by the culture with G418 (400 μg/ml). As shown in Figure 1, we could identify the ras-17Asn transformed HS-5 cells, showing negative Ras protein expression as determined by Western blot analysis with anti-Ras specific antibody.

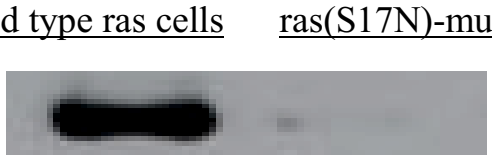
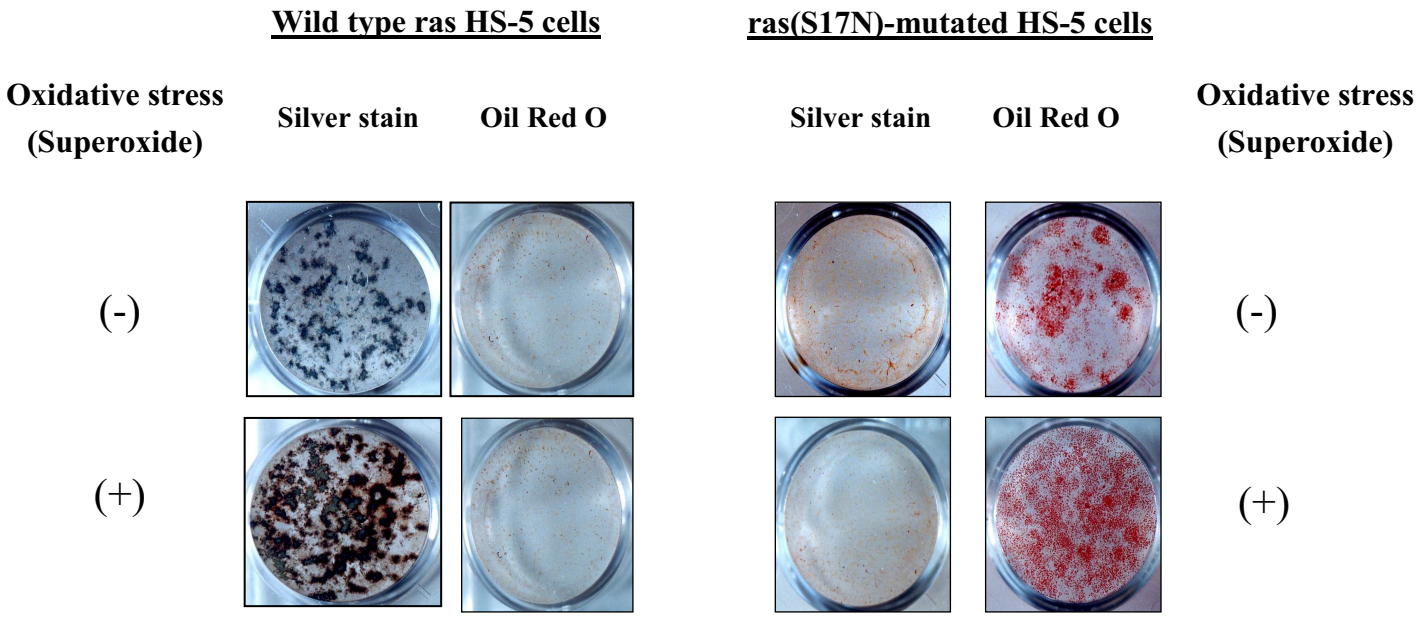


Fig 1. Transformation of wild type ras HS-5 cells to ras dominant negative HS-5 cells

Oxidative stress induced bone and fat cell differentiation, respectively, in wild type ras and ras(S17N)-mutated HS-5 cells

Studies were started with a 2 x 2 factorial design. Wild type ras and ras(S17N)-mutated HS-5 cells ($1x10^5$ cells/well) suspended in 24-well plates were treated with and without exogenous O_2^- produced by the catabolization of hypoxanthine (10 mM) with xanthine oxidase (250 μU/ml) for 2 hours. After washing out hypoxanthine and xanthine oxidase, the cells were cultured in DMEM medium for 15 days. Results showed that the wild type ras HS-5 cells differentiated into mainly bone nodules as shown by von Kossa staining but not lipid-containing cells as demonstrated by Oil Red O staining (left panel, Figure 2A). In contrast, the ras(S17N)-mutated HS-5 cells differentiated into mainly lipid-containing cells as demonstrated by Oil Red O staining but not osteogenic bone nodules as shown by von Kossa staining (right panel, Figure 2A). The microscopic morphology (100 X magnification) shown in Figure 2B was compatible to those gross pictures shown in Figure 2 A.

A. Gross observation of cell differentiation



B. Microscopic observation of cell differentiation

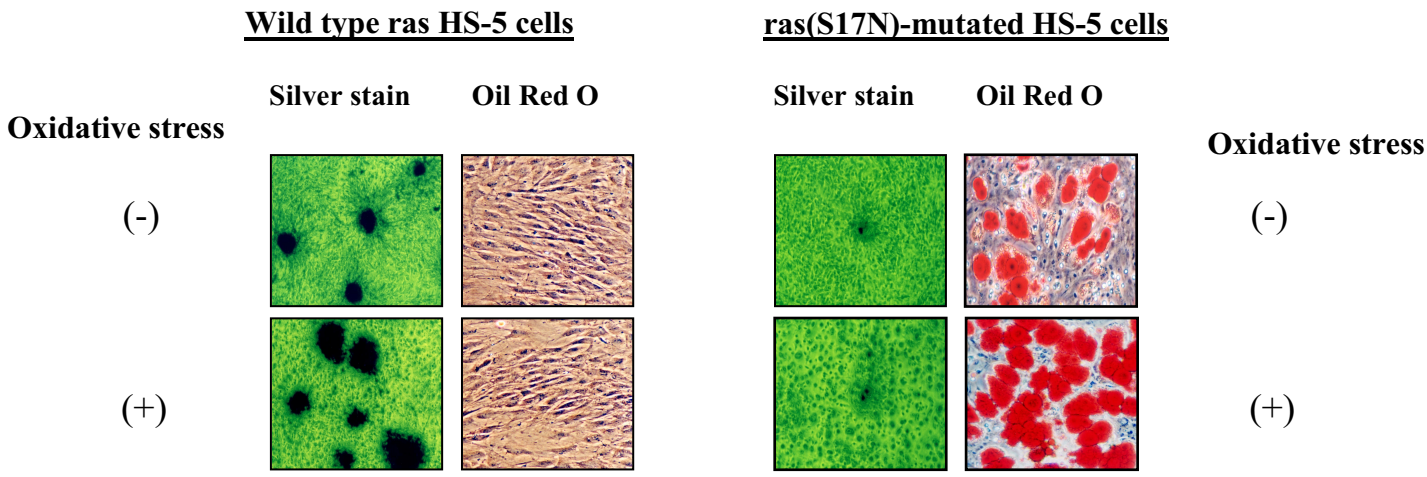


Fig 2. Influence of oxidative stress on wild type ras and ras(S17N)-mutated HS-5 cell differentiation (100 X magnification)

Oxidative stress mediated different signals in wild type ras and ras(S17N)-mutated HS-5 cells.

To explore the oxidative stress-mediated signal transductions in wild type ras and ras(S17N)-mutated HS-5 cells, we assessed changes of the signaling molecules in the cells after stimulation of exogenous O_2^- . It was found that O_2^- elicited a rapid Ras and ERK activation within 6 hours, induction of osteogenic transcription factor CBFA1 in 24 hours, expression of osteocalcin in 7 days and bone nodule formations in 15 days in wild type ras HS-5 cells (left panel, Figure 3). In contrast, O_2^- did not elicit ERK or CBFA1 activation, but induced adipogenic transcriptional factor C/EBPα expression, followed by lipoprotein lipase expression and positive staining of mature fat droplets (right panel, Figure 3). Scavenging of O_2^- by superoxide dismutase (SOD; 500 U/ml) specifically suppressed O_2^- induced osteogenesis in the wild type ras cells, indicating that O_2^- directly involved in the signal transduction for osteogenesis (data not shown).

Oxidative stress enhanced TGFβ and IGF-I production by wild type ras and ras(S17N)-mutated HS-5 cells, respectively

The oxidative stress starting with addition of the exogenous O_2^- produced by the reaction of xanthine oxidase (250 μU/ml) with hypoxanthine (10 mM) promoted TGFβ production by wild type HS-5 cells ($p< 0.01$, t test; Figure 4). The oxidative stress did not enhance TGFβ production, but enhanced IGF-I production by ras(S17N)-mutated HS-5 cells (Figure 4).

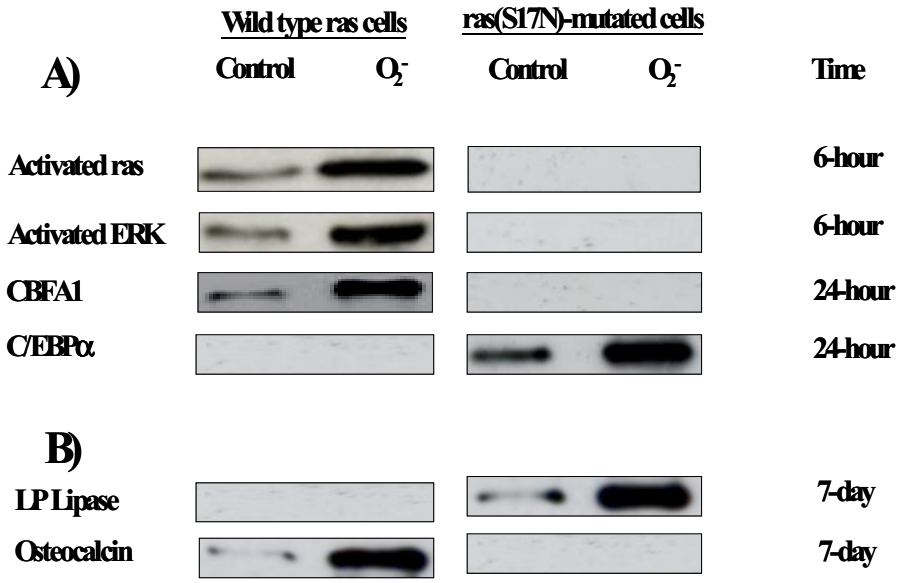
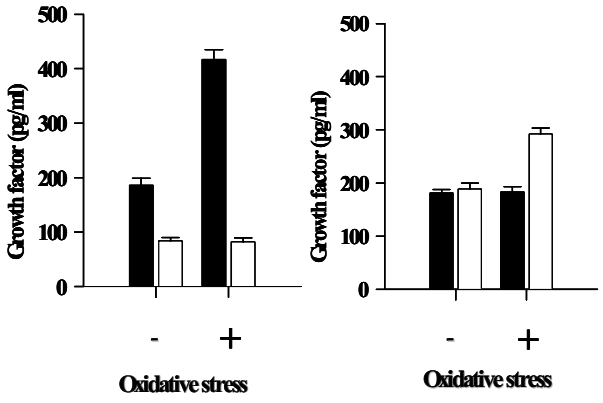


Fig. 3 Oxidative stress mediated different signals in wild type and ras (S17N)-mutated H-S-5 cells.
A): Signal transduction under oxidative stress ; B): Bone and fat cell maturation markers.



Wild type ras cells treated with superoxide for 2 hours significantly increased TGFβ production (black bars) in 3 days ($p < 0.01$; $n=6$); but for ras(S17N)-mutated cells superoxide enhanced IGF-I production (white bars; $p < 0.05$; $n=6$).

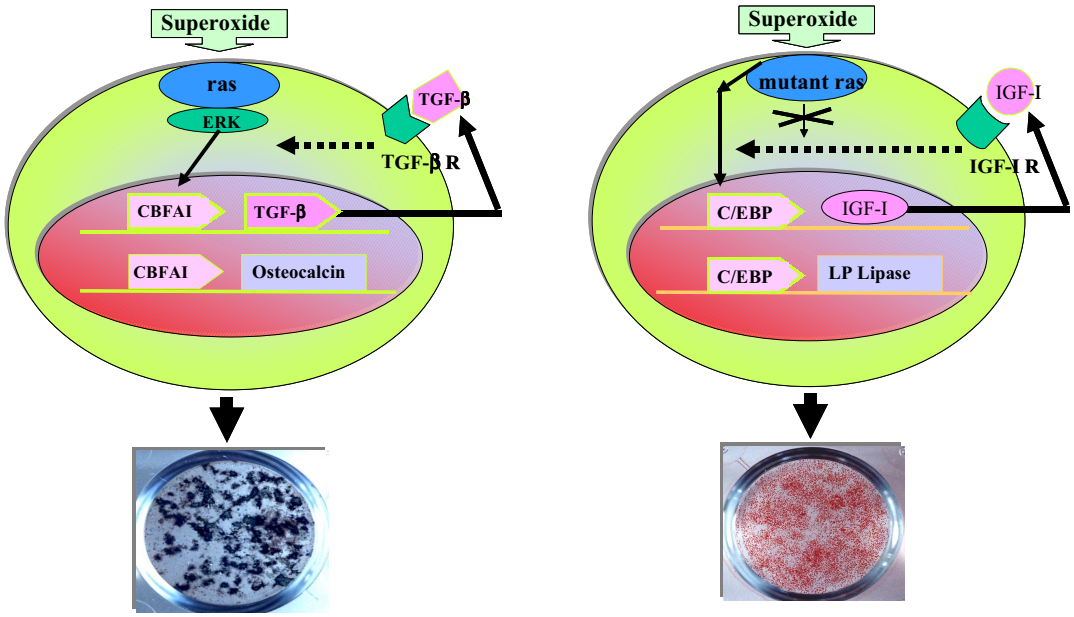
Fig. 4. Oxidative stress enhanced mesenchymal cell growth factor (TGFβ & IGF-I) production.

DISCUSSION

This study shows that oxidative stress can enhance the differentiation of wild type HS-5 mesenchymal cells to bone nodule formations via ERK activation in hours, followed by osteogenic transcription factor CBFA1 expression and osteogenic growth factor TGFβ production in hours to days, and matured differentiation marker osteocalcin expression in days to weeks.

By contrast, oxidative stress enhaces the ras(S17N)-mutated HS-5 cell differentiation to fat cells via induction of adipogenic transcription factor C/EBPα, followed by adipogenic growth factor IGF-I production, and matured differentiation marker LP lipase expression. These findings comprise the first evidence demonstrating that an appropriate oxidative stress promotes bone or fat cell differentiation depending on ras gene expression.

Further studies to promote bone growth from mesenchymal stem cells may be made possible via regulation of redox reaction and signal transduction. Mechanisms for the possible regulation of bone and fat cell differentiation from different mesenchymal cells could be depicted as follows:



CONCLUSION

We found that oxidative stress had different effects on bone and fat cell differentiation in different Ras expressing cells. The mechanism for the oxidative stress-mediated bone cell differentiation was demonstrated via the signal transduction pathway from Ras Raf-1 ERK CBFA1 osteocalcin in wild type HS-5 mesenchymal cells, while the differentiation of fat cells was shown through C/EBPα LP lipase in ras (S17N)-mutated HS-5 cells. This study model may be applicable for making bone cells from not only bone marrow stem cells, but also human umbilical cord blood stem cells or embryonic stem cells for bone tissue engineering.

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評　　語

本計畫爲組織工程（Gewtic Engineering），利用人類骨髓間質細胞株，經 ras 基因處理，或氧化與抗氧化調控間質細胞之分化，結果顯示間質細胞經十五天轉換成骨質細胞，而於 ras 突變株於氧化壓力下成爲脂肪細胞，並經分生方法証明骨質細胞有 Osteocalcir，而脂肪細胞有 Lipase 之基因表現，本計畫具創見性及學術價值。